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Food Science

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SPECIAL VOLUMES

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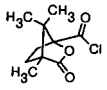

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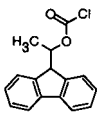

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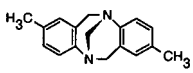

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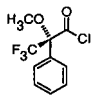

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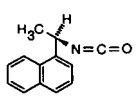

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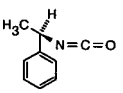

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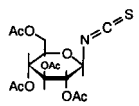

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APPLICATIONS OF CHROMATOGRAPHY AND ELECTROPHORESIS IN FOOD SCIENCE

CONTENTS

APPLICATIONS OF CHROMATOGRAPHY AND ELECTROPHORESIS IN FOOD SCIENCE

In articles with more than one author, the name of the author to whom correspondence should be addressed is indicated in the article heading by a 6-pointed asterisk (*).

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CHROM. 24 489

Foreword

Food and food products represent a specific area of analysis. The materials to be analysed always represent very complex mixtures and, consequently, most analytical approaches require specific sample treatment. Whereas in the biomedical field the analyst is always struggling with the scarcity of material for analysis, in food science this is not the case. On the other hand, many types of food analyses are done by means of standardized methods which, with the advancing knowledge of nutrition physiology, are likely not to offer adequate information. It is just here where separation methods are purposefully applied. In this volume we have tried to present current chromatographic and electrokinetic methodologies that can offer a deeper insight into the categories of compounds present in foods and food products. As far as the nature of the analysed compounds is concerned, they either may be inherent to the food samples, *e.g.*, proteins in milk and milk products and proteins in meat, or may represent minor but important constituents of food, such as vitamins, pigments and toxins, may be present in food as a result of previous treatment of fauna and flora, *e.g.*, with pesticides or drugs. A specific, analytically very different area is represented by compounds causing flavours and odours.

We have tried to assemble reviews on all these topics within a single volume of the *Journal of Chromatography* in order to supply the reader with a cohesive source of information that at present is widely scattered throughout the literature. We are aware that we have not covered the field absolutely without any gaps, simply because we were unable to find experts ready to prepare a sufficiently generalized overview. In those instances where the separation procedures reviewed are applicable to several categories of foods, we have tried to emphasize the differences in analytical approaches to individual categories.

Of course, as with other topical issues and volumes of this journal, we would be obliged to our readers for any comments regarding this volume as they may be helpful in our future work.

Prague (Czechoslovakia) Zdenek Deyl

Journal of Chromatography, 624 (1992) 3–9
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 036

Review

Sample preparation for chromatographic analysis of food

Michael J. Lichon

Department of Plant Science, University of Tasmania, GPO Box 252C, Hobart, Tasmania 7001 (Australia)

ABSTRACT

Sampling, homogenisation and sample preparation prior to chromatographic injection of food analytes are designed to enhance accuracy and precision. The reduction of inherent errors introduced by these steps requires the analyst's attention as a matter of course. Methods and examples of minimising errors in each step are reviewed.

CONTENTS

1. INTRODUCTION

Almost without exception, food is a complex inhomogeneous mixture of a bewildering array of chemical substances. The isolation and measurement of individual chemical compounds in food represents a difficult task. Chromatography is a powerful technique of separation and identification, yet rarely is it possible to directly load a syringe with a food matrix and inject to obtain a sensible result. Perhaps surprisingly, it is not rare to find analytical methods published with precision data reflecting repeated direct injections of standard solutions. This tells the reader little about the practicality of the method to real world samples. Procedures for preparation of the sample should be developed, evaluated and published as an integral part of any analytical method.

There are three steps involved in sample preparation for chromatographic analysis of foods: (1) *sampling*, obtaining a sample for the laboratory; (2) *homogenisation* of the laboratory sample to enable the taking of test portions; and (3) *sample preparation*, physical and chemical manipulation of the test portion prior to injection of the analyte fraction into the chromatograph. It should be appreciated that elements of these steps may occasionally occur in the reverse order or as combined operations. The fourth and final step of the analysis is the chromatography. Paradoxically, although the purpose of each of the three steps is to increase the accuracy and precision of the analysis, each step also introduces inherent errors. The error contributions of these steps for a typical food analysis scheme are shown in Fig. 1. Analyte concentration is limited at one end by detection limit and at the other by overloading of preparation stages or the chromatograph by either analyte or matrix. The significance of the contribu-

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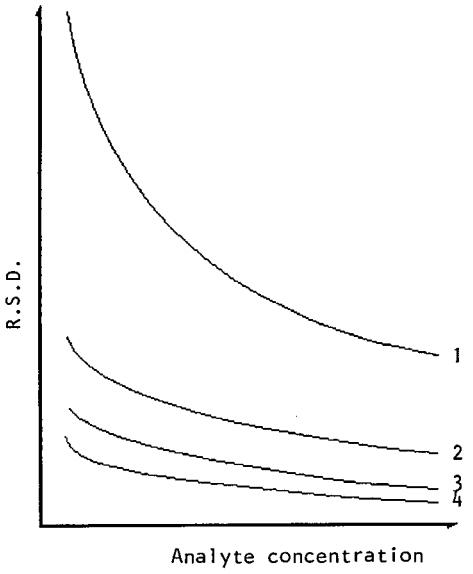

Fig. 1. Typical relative standard deviation of error components for an inhomogeneous matrix. 1 = Sampling; 2 = homogenisation; 3 = sample preparation; 4 = chromatography.

tions of these steps to the total error for the analysis are mathematically described by the relative standard deviation (R.S.D.) relation:

$$\text{R.S.D.}_{\text{total}} = [\text{R.S.D.}^2_{\text{sampling}} + \text{R.S.D.}^2_{\text{homogenisation}} + \text{R.S.D.}^2_{\text{sample preparation}} + \text{R.S.D.}^2_{\text{analytical}}]^{\frac{1}{2}} \quad (1)$$

From the equation it is clear that if any one of the contributing factors is significantly greater than the others, it is futile to attempt to reduce any of the other contributors, as the total error will be disproportionately dictated by the dominant factor being squared. A good example is aflatoxin analysis, where as long as the sampling error contributes 90% of the error there is little incentive to improve the analytical precision [1].

It may often be clear to the experienced analyst what the approximate proportions of the contributing errors are of the total for a familiar analysis. If this is not so, then these may be defined by rigorous assays of replicates and recoveries testing the effects of each successive step of the analysis. This review paper has been divided into three sections to consider problems encountered in each of these respective steps.

2. SAMPLING

There has been great concern amongst analysts over the validity of analytical methods. Attempts to rigorously define precision and accuracy of methods include measures such as international collaborative trials. For all the benefits of implementing such expensive measures there is one important oversight; the issue of sampling is not examined. Experience demonstrates that sampling can often be the greatest source of error in chemical analysis, particularly for food matrices (Fig. 1).

> "The classic example of incorrect sampling procedure and its ridiculous consequences is given by the fable of the blind men and the elephant. The consequences are sometimes no less ridiculous for incorrect chemical sampling" (W. J. Blaedel and V. W. Meloche [2]).

Undergraduate texts often form the basis of analysts' future attitudes. The spectrum of emphasis on sampling ranges from serious but brief mentions [3–5] through a good treatment, but at chapters at the end of the book [2], to an integral treatment from the beginning [6].

The best method of sampling in a given situation will depend on such issues as: what information is sought, resources available, accessibility of the target population, is the population heterogeneous? and if so is the variation general, localised or stratified?, required turnaround time, perishability of the food and the analyte, should the population be sampled critically or representatively, randomly or systematically? What are the criteria for acceptability? Are samples to be pooled or replicated? Should analysis be performed separately on different portions of the sample, is the surface to be included in the bulk? What monitoring should occur to prevent contamination and abuse?

Take the example of nutritional analysis of peas: sample variables include size distributions, position of individual peas in their pods, height of pods up the individual vine, individual plant genetics, cultivar, time of planting, efficiency of pollination, watering history, soil type and underlying geology, previous crop history, soil fertilisation, crop maturity, disease and pestilence attack, length of time, handling and storage conditions since harvest. Are the peas to be cooked? If so, how and for how long? Is the analysis to represent nutritional data for a locality, or national database?

These issues are discussed to varying degrees in the sampling literature, are mostly self explanatory or specific to situations. In the latter case discussion is usually found in sections of literature specific to the situation, or remain unrecorded know-how of specialists. These specialists must be encouraged to include such details in their publications.

Literature concerning sampling is well dispersed and generally not easy to locate. A useful survey [7] lists over 60 references of possible use to food analysts, some general references [8–12] being recommended. Several others included various mathematical treatments [13–15] of both general and specific problems. A mathematical consideration [16] of chemical analysis was not included, presumably because it concentrates on assessment of data quality rather than the practical aspects of sample planning. The American Chemical Society has published guidelines [17] regarding sampling. International Standards are complete for sampling of fruits and vegetables [18], meat [19] and oilseeds [20].

More recent discussions of sampling [21–24] have attempted to integrate sampling approaches with laboratory practices, sample characteristics and analytical problems.

The importance of sampling has recently been highlighted by problems encountered in analysis of aflatoxins in peanuts. Several workers have written papers specifically addressing this type of sampling situation [1,25–28]. In these cases, of highly inhomogeneous distribution of analyte in the matrix, the proportion of total analytical error attributable to sampling is commonly greater than 90%.

All too often the analyst has little influence over the taking of the sample. A widening of appreciation of the importance of sampling may serve to rectify this problem. Ideally the analyst should appraise the problem and take the samples personally. Failing that, the analyst should endeavour to thoroughly brief the sampler as to the most appropriate methods for each situation. A specific instance of such difficulty concerned an untrained sampler being assigned to learn about the traditional indigenous foods of Australian Aboriginals. He was also briefed to collect specimens for nutritional analysis, but was little influenced by the analysts. Constraints apart from the lack of training were the hot climate, lack of refrigeration, necessity of taking pocket-size samples, and remoteness from the laboratory, some 3000 km and several days freight away. All of these factors contributed to the degradation of sample integrity. Many of the samples analysed consisted, for example, of three individual thawed fruits totalling 15 g. However while the limitations of the nutritional assays on such unrepresentative samples are obvious, the data are surely indicative and as such useful in this context [29–31], where previously nothing was known of the nutrient content of the foods.

3. HOMOGENISATION

The complex structure and composition of food substrates necessitates homogenisation prior to most chromatographic analysis. Variable texture, structure and viscosity, and the presence of immiscible phases, hygroscopic or hydrophobic matter all contribute to the difficulty of this operation meeting with success. The observation that collaborative test results for food materials often show greater coefficients of variation than other matrices [32] is therefore not surprising.

Problems encountered with sampling, particularly for semi-micro combustion analysis [33] led to the author's investigations concerning homogenisation methods used for food samples [34]. This paper surveys nine conventional methods and three cryogenic methods of homogenising numerous food samples, condensed into seven categories of matrix type. Several methods were subjected to more rigorous examination. Other papers have considered more limited ranges of methods and foods [10,35–37]. For assays using test portions of around 1 g several of the conventional methods prove satisfactory with compatible matrices [34]. Many method–food category combinations proved to be incompatible, some unexpectedly so. For assays using smaller test portions of requiring stringent homogeneity of very heterogeneous foods the cryogenic treatments proved well worth the extra effort after a conventional pretreatment and freeze-drying. This dual treatment reduced particle sizes to below 60 μm (97% below 10 μm) for one of the most difficult matrices with sufficient mixing to take reproducible test portions of 1 mg. The average R.S.D. of micro-combustion protein assays for a range of foods was 1.33%, performed on test portions of 2–5 mg [34]. The number of particles included in

each test portion was approximately 10^5.

The required size of the test portion and the sample's characteristics will dictate the degree and type of homogenisation required. If several different assays are to be performed on a sample then whichever has the most stringent requirements will often dictate the homogenisation requirements. Experience shows that it is often prudent to sequentially use two homogenisation techniques. It may be desirable to split the sample after an initial wet-basis homogenisation treatment; analysing the first part for labile vitamins directly; further rigorous homogenising after freeze-drying before subjecting the second part to other analysis. A generalised scheme for homogenisation of samples for nutritional analysis is shown in Fig. 2. The scheme includes approximations for quantities and particle sizes at each stage, and what types of assays are amenable to the products of each stage. However, it must be reiterated that each sample will have different characteristics which will require different homogenisation treatments. The main conclusion that should be drawn is that each sample should be homogenised by methods that have proven effectiveness with the particular matrix, either from experience, literature or by experiment, to a degree that meets the test portion requirements. This must be confirmed by the precision of replicate assays.

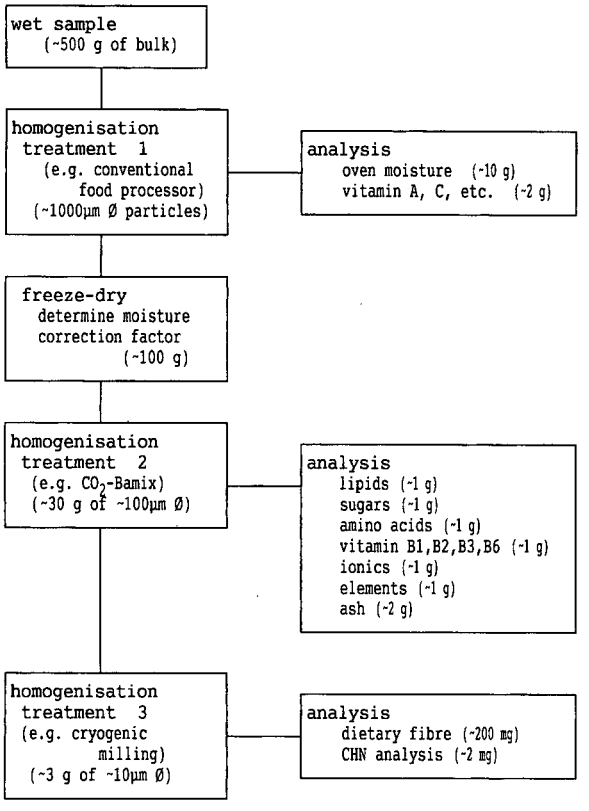

Fig. 2. A sample homogenisation scheme for nutritional analysis of food. $\varnothing$ = Diameter.

There are two functions of homogenisation, reduction of particle size and mixing. Reduction of particle size involves cutting, shattering and shearing. The various devices achieve these in different ways to differing degrees. This necessitates judicious choice of homogenisation methods that have demonstrated applicability for use on particular matrices. The efficacy of a method may be observed by microscopic examination or sieving of the product. The importance of particle size reduction is intuitive; quantifying this statistical notion is more difficult. A simplified treatment with graphed relationships [11] is recommended reading for non-mathematical analysts.

Mixing may be more difficult to achieve and examine. Experience suggests visual inspection of colour and texture is very useful, but not necessarily rigorous, especially in the case of a sample consisting of components of similar appearance. There are several means that may prevent adequate mixing: classification, agglomeration and phase separation. Causes include particle shape differences, density differences, electrostatic charging, disruption of stable structures maintaining surface tension, destruction of encapsulating structures, and various hydrophobic–hydrophilic interactions. Typical examples include oil separation in finely ground nuts and classification of whole grain flour. The only way of avoiding such problems is selecting appropriate homogenisation methods through experience, learning, but just as often by intelligent trial-and-error experimentation. The combined effect of reduction and mixing may be examined by performing assays on replicate test portions. For this examination it may be prudent in some circumstances to run simple, cheap assays rather than use the actual target assay. Another method of potential is statistical image analysis of the homogenised test portions under the microscope. It may be necessary to colour-label some components prior to treatment to aid differentiation.

The degree of homogenisation may affect the accuracy as well as the precision of the analysis; in ways that may not be revealed by recovery data. A typical case is the accessibility of the food matrix to enzymatic digestion. The efficiency of enzymatic digestion is proportional to the surface area (or degree of homogenisation) of the food substrate; that is, inversely proportional to particle size. Large particle size may inhibit enzyme access to the whole of the food, specific portions may be encapsulated and not be available for subsequent extraction; such problems have been encountered in thiamin [38], lipid [39] and dietary fibre [40] analysis. Efficient thiamin extractions were only possible after digestion of finely homogenised foods. In the second example, digestion of a well homogenised substrate was required for complete release of lipids; but if the sample was too finely ground, problems with emulsion formation hampered the efficiency of the subsequent liquid–liquid extraction. In contrast, Englyst dietary fibre determinations [40] on poorly homogenised samples give spuriously high results due to incorporation of undigested starch in the fibre fractions. Analysis of total dietary fibre by the AOAC method [41] has different problems: if the sample is too finely ground then there is the risk of low results by losing fibre through the 90-μm porosity filter, even with the use of filter aids; if the sample is too coarse then there will be high results from insufficient enzymatic digestion of other components, and a loss of precision from poorer test portion sampling of larger particles.

4. SAMPLE PREPARATION

Sample preparation includes any operation performed to the test portion prior to injection into the chromatograph; weighing, dilution, cleanup, extraction, digestion, purification, separation, derivatisation etc. ... Descriptions of which are usually included in publications, although frequently lacking background information, "tricks of the trade" finer detail, and rigorous error analysis.

Careful thought and often some background research is required to decide what parts of the sample are to be analysed. For example, is fresh produce to be washed prior to pesticide analysis? If so, how? What is the "edible portion"? Examples of such separations include removal of outer leaves, peels and pips from fresh fruits and vegetables, removal of bones and trimming of excess fat from meat, exclusion of brines from canned vegetables but inclusion of liquid from canned fruit. Many such choices are subject to debate, such as the inclusion or otherwise of seeds in a sample of blackberry jam. It may be desirable to analyse both portions. In any case it is standard practice to weigh the separate portions, analogous to the determination of moisture when drying a sample.

Several authors have emphasized that for nutritional evaluation it is desirable to prepare the food as it is consumed [10,21]. What are the customary methods and times of cooking of meals? What constitutes complete preparation of powdered soups and hot beverages. An instance that comes to mind is the analysis of such beverages for vitamins B_1 and C, where the assay of the powder is assumed to be the measure of dietary intake [42,43]. In reality, these vitamins degrade significantly when the powders are stirred into boiling water, especially if the water has been sterilised by iodine agents, as is recommended practice in this context.

The first and practically universal step in the manipulation of the test portion is the weighing step. Fortunately the precision of modern balances is commonly six significant figures or better. Judicious choice of balance can maintain this precision for a large range of test portion size, typically from 10 g down to 1 mg for food analysis. However the analyst must ensure the operation is performed accurately, paying particular attention to eliminating electrostatic charging and moisture variation of food samples. These same errors must be considered when drying samples and determining moisture correction factors. Once dry, samples should be stored in a desiccator.

A frequent operation contributing to the sample manipulation error is the volumetric dilution. For headspace sampling, the size, pressure, equilibration time and temperature of the space are all critical. Volumetric errors are inherently orders of magnitude greater than those for mass measurements.

Digestion, extraction and derivatisation should all be quantitative. Efficiency may be enhanced, for example by application of microwaves [44], but only if sample integrity is maintained. Confirmatory tests should be used in any doubtful cases. A typical simple test is the testing for residual starch with

drops of iodine to confirm completion of amylase digestion [40].

The methods of ensuring good recoveries while using absorption columns prior to liquid chromatography are straightforward. The use of preconcentration methods in gas chromatography [45] can be fraught with complications of volatility differences, reactivity and adsorption. These issues are generally adequately discussed in methodology papers alongside chromatography details.

Degradation of the sample and analyte integrity may take place at any stage, from the taking, transport and storage of the sample, drying, homogenisation and sample preparation to the injection of the manipulated test portion. Addition of contaminants, exposure of samples to heat, warmth (microbiological activity), moisture, oxygen, visible and ultraviolet light, reagent fumes can all compromise accuracy. These problems are considerable in vitamin analysis —consider some examples:

Riboflavin is sensitive to ultraviolet light. Vitamins A, B_6, D, E and folic acid are sensitive to light. Laboratory manipulations are usually performed using low-actinic glassware and preferably in the dark [38,46,47].

Ascorbic acid is particularly sensitive to degradation by oxidation, especially when exposed to atmospheric oxygen, heat or high pH. Analysis schemes aim to reduce manipulation and turnaround time to an absolute minimum, making use of various stabilising agents. The tenfold variation in ascorbic acid found in *Terminalia ferdinandiana*, a native plum found in northern Australia rich in this vitamin [29,30] is at least partly due to degradation during lengthy transport on different occasions. A less obvious hazard is contamination by traces of copper, which catalyses the oxidation reaction.

Introduction of metal contamination, by the homogenising device (for example), may be serious beyond the simple raising of metal content. The presence of metal may promote reactions compromising sample integrity, as mentioned above, but may also interfere with extraction, cleanup and enzymatic digestion steps of sample preparation procedures.

Contaminants may be introduced by reagents. The development stages of a method for enzymatic digestive release of lipids [39] revealed that several commercial enzyme preparations contained unacceptably significant amounts of ether-extractable contaminants.

Contamination and the potential complications in sample preparation procedures are highlighted by the gas chromatography of alditol acetates in the Englyst dietary fibre determination. Plasticisers may contaminate food samples at literally any point from the farm to the chromatograph. Pure samples of ubiquitous plasticiser contaminants were found to chromatograph at similar times to some of the analytes [48], but could be resolved from analyte peaks by capillary columns. More recent investigations found that exposing plasticisers to the derivatisation procedure used to form alditol acetates yielded multiple and broad peaks that potentially interfered with the analytes [40]. This is an additional artifact caused by the sample preparation technique fundamental to the analysis. A recent monograph [49] deals specifically with such analytical artifacts, with considerable attention to problems with gas chromatography–mass spectrometry, a technique widely regarded as definitive with respect to analyte specificity.

All elements of the gamut of test portion manipulations performed are potentially significant error contributors. The consistent use of observation, replication of test portions, recoveries and reference materials should highlight problem areas. These may be reduced, or at least quantified, using the skill of the analyst.

5. CONCLUSIONS

The analyst wishing for accuracy and precision must focus on all elements in each of the four steps of analysis; sampling, homogenisation, sample preparation and analytical technique. Critical examination should reveal weaknesses where sample integrity may be compromised. The greatest effort should be expended to reduce contributions in the error-dominating steps. Authors should be encouraged to include all experimental details of the first three steps in their publications.

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Review

Advances in planar chromatography for the separation of food lipids

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ABSTRACT

A survey of the advances in planar chromatography for the separation of food lipids is presented. Techniques of planar chromatography [densitometry, preparative thin-layer chromatography (TLC) and TLC with flame ionization detection, etc.] together with applications from the areas of dairy, marine and plant lipids are discussed. Additives such as lecithins (emulsifying agents) are also considered.

CONTENTS

1. INTRODUCTION

Chromatography performed in open systems, such as on thin-layer chromatograms or on Chromarods, represents a dynamic and versatile alternative route to column liquid chromatography for analyses for food lipids. The advances in this technique over about the last 5 years in the separation of food lipids is discussed in this review.

Planar chromatography [1] on a silica matrix has traditionally been the method of choice for the separation of lipophilic substances. Since its appearance in the late 1950s it has been the most practical method capable of distinguishing between lipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), etc.]. Lipid analysis prior to planar chromatography was an imprecise and tedious task, performed by methods such as iodine value determination (measure of unsaturation) and elemental analysis for nitrogen, phosphorus or sulphur.

During the past decade it has been gratifying to see that planar chromatography, *i.e.*, thin-layer chromatography (TLC), has maintained its position

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as one of the major separation techniques in the area of lipid analysis [2,3]. The improvements in TLC scanners, equipped with, *e.g.*, zig-zag scanning functions, for *in situ* evaluation of planar chromatograms has led to enhanced accuracy in quantification and ease of operation (computer control). Sample requirements for quantification in TLC have been reviewed by Poole *et al.* [4].

As a consequence of the above, quantitative planar chromatography can no longer be claimed to represent an inexpensive analytical technique. Reproducible sampling devices and computer-controlled *in situ* scanners place TLC in the same cost range as high-performance liquid chromatography.

It is beyond the scope of this review to discuss the benefits of planar chromatography in comparison with other analytical techniques, or the latest instrumental developments in the field of TLC which have been extensively reviewed by Erdelmeier and König [5].

Analyses for lipids in foods by planar chromatography serve mainly to determine the lipid class composition [6]. Lipid classes can be divided into two sub-groups [7]: the complex, polar lipid classes and the simple, neutral lipids. The former yield at least three different substances on hydrolysis, such as free fatty acids, glycerol and phosphorylcholine from PC. The latter yield no more than two different groups on hydrolysis, *e.g.*, free fatty acids (FFA) and glycerol from triacylglycerols. The polar lipids are ubiquitous structural components in the membranes of all plant and animal tissues and they can also serve important functions as multi-purpose food additives.

Phospholipids contribute to the flavour and taste of cereals, legumes and meats. As natural phospholipids contain mono-, di- and polyunsaturated fatty acyl chain, they are susceptible to oxidative deterioration which might result in phospholipid residues with off-flavors. Hydrolytic enzyme systems present in foods, *i.e.*, in the cellular membranes, will degrade phospholipids, producing free fatty acids and monoacyl phospholipid residues [8]. The level of free fatty acids, easily monitored by TLC, can be used as a measure of food deterioration.

Fish meats and oils, rich in polyunsaturates, are especially susceptible to auto-oxidative molecular degradation, which eventually leads to relatively low-molecular-mass carbonyl compounds with unpleasant smells. Lipid deterioration can be measured by monitoring aldehydic peroxidation (secondary oxidation products) with TLC in conjunction with densitometry [9].

2. FUNDAMENTALS OF LIPID ANALYSIS BY TLC

The fatty acid composition (O-acyl or N-acyl lipophilic chains) of polar and neutral lipids is usually determined (in the form of methyl ester derivatives) by gas chromatography (GC) and the cascade of molecular species, within defined underivatized lipid classes [neutral (NL) and polar lipids (PL)], is generally monitored by reversed-phase high-performance liquid chromatography (HPLC) or by high-temperature GC (only NL) between 280 and 360°C. Thus, planar chromatography is mainly utilized in normal-phase (silica matrix) applications and in group separations according to degree of unsaturation (silica matrix, impregnated with silver ions), *i.e.*, argentation chromatography. Both of these methods have also been used in preparative applications. A chromatographic description (R_F values) of some food lipids is given in Table 1.

Quantification of separated lipids in planar chromatography is performed either by *in situ* measurements (densitometry [10], radiochromatography [11]) or by tedious gravimetric methods which

TABLE 1

R_F VALUES OF SELECTED FOOD LIPIDS ON TLC IN VARIOUS SOLVENTS (AFTER REF. 40)

Solvents: 1 = chloroform–methanol–water (65:25:4); 2 = chloroform–methanol–acetic acid–water (50:30:8:4); 3 = chloroform–methanol–28% (w/v) ammonia solution (65:25:5).

Lipid component	R_F × 100 in solvents		
	1	2	3
PI	23	47	11
PC	33	31	33
PE	62	83	41
PS	15	55	5
PA	74	–	5
SPH	16	18	22
DGDG[a]	62	–	–
MGDG[b]	77	–	–

[a] Digalactosyl diglyceride.
[b] Monogalactosyl diglyceride.

include scraping off the spots and subsequent extraction of substances from the silica matrix. Flame ionization detection (FID) measurements can be used in confunction with chromatography on silica-coated quartz rods, *i.e.*, Chromarods. This methodology is called TLC–FID, or simply Iatroscan after the instrument generally utilized (Iatron, Japan). Detector linearity with TLC–FID methods is better than that obtained in densitometric measurements, which is the major justification for this principle of planar chromatography.

3. DENSITOMETRY

Charring of spots on thin-layer chromatograms by, *e.g.*, sulphuric acid and heat has traditionally been the route to detection and is still widely used. However, this procedure can generate a high background signal when used in conjunction with scanning densitometry, partly because extraneous particles (dust) present in the air adhere to the silica surface of the plate, becoming charred together with the separated compounds, Further, this method is destructive and separated/purified lipids cannot be recovered.

Another way to detect phospholipid classes on planar chromatograms for further densitometric evaluation is to use a lipophilic fluorescent reagent in the mobile phase [12]. By this elegant procedure phospholipid amounts down to 0.29 μg could be detected and measured.

The problem of revealing lipid spots on TLC plates has been addressed by Olsson *et al.* [13]. The utility of iodine vapour for detection and subsequent densitometry was investigated. It was shown that iodine vapour partitioned reversibly to all lipid samples rested, even fully hydrogenated fish oil and hydrogenated soybean PC. The results indicated that the partition of iodine to lipid spots on the TLC plates was due to several factors, of which unsaturation seemed to be the most influential. Other factors were the nature of the acyl carrier and the average chain length of the acyl group. Further, the rate of elimination of iodine from spots appeared to be mostly dependent on the lipid class, rather than the degree of unsaturation. It was found that 15–20% of the iodine remained in natural soybean PC 7 days after removal of the plate from the iodine chamber.

It might be speculated that this procedure could be utilized in quantitative analysis, even though a steady state in the elimination of iodine does not seem to occur.

3.1. Two-dimensional TLC

Two-dimensional planar chromatography represents an efficient utilization of TLC plates in terms of resolution of complex mixtures found in foods and food additives. Two-dimensional chromatograms contain a large amount of information and, used in conjunction with a reproducible integration system, this information can be made available. The drawbacks of the technique are that the sample capacity is limited to one spot per plate and that quantitative *in situ* evaluation is cumbersome.

In a paper by Lam and Sequera [14], polar lipid classes [PC, PE, PI and phosphatidic acid (PA)] of soybean were model substances in the exploration of quantitative two-dimensional (2D) TLC, utilizing a Shimadzu CS 9000 U scanner in conjunction with QuantaScan 2D Analysis software. The results clearly showed that 2D quantification could only be used when the data were normalized to an internal standard. Even if the variations in the normalized 2D determinations were worse than those obtained in the regular densitometric evaluation of linear lanes of spots, it was a major accomplishment compared with, *e.g.*, gravimetric evaluation, which otherwise would have had to be employed.

Quantitative densitometric evaluation of 2D planar chromatograms seems to require considerable further development before it could be considered a convenient and reliable method. With the recent developments in computer-controlled image analysis, such tools should soon become available.

3.2. Optimization

Optimization of chromatographic performance has traditionally been performed on one variable at a time (stationary phase, ionic strength, pH, etc.), *i.e.*, with univariate methods. As essentially all variables interact in the complex chromatographic process, univariate methodology often fails to locate true optimum conditions (optimum spot resolution, optimum response, etc.) [15]. Optimization based on multivariate methods [16] can handle related variables in a rational and, thereby, time-efficient way. Optimization in planar chromatography has been reviewed by Siouffi [17].

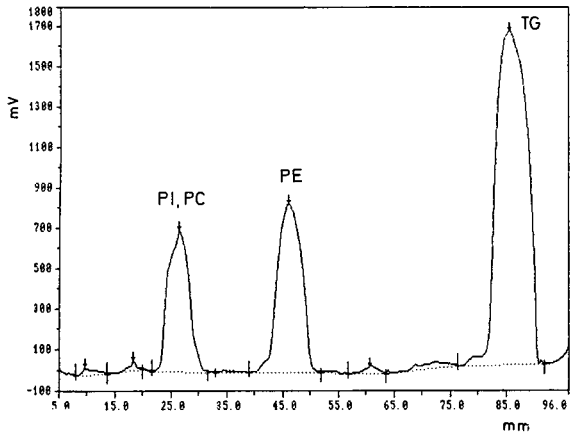

Fig. 1. Densitogram of a standard mixture (PI, PE, PC and TG) developed with original system; chloroform–methanol–acetic acid–water (68:22:6:4, v/v). (Ref. ref. 19, with permission).

Emulsifying agents, such as lecithins [18], are used as additives by the food industry in low-fat spreads and ice cream. An optimized TLC method was developed by Olsson *et al.* [19] for the analysis of the major lipid classes of natural lecithins, utilizing factorial design and multivariate optimization methods. An optimum separation, within the chosen experimental domain, between PI, PC, PE and the triacylglycerols (TGs) was obtained in 21 experiments. The results of this investigation are illustrated in Figs. 1 and 2, where the optimized TLC system (Fig. 2) is shown in comparison with the non-optimized original system (Fig. 1).

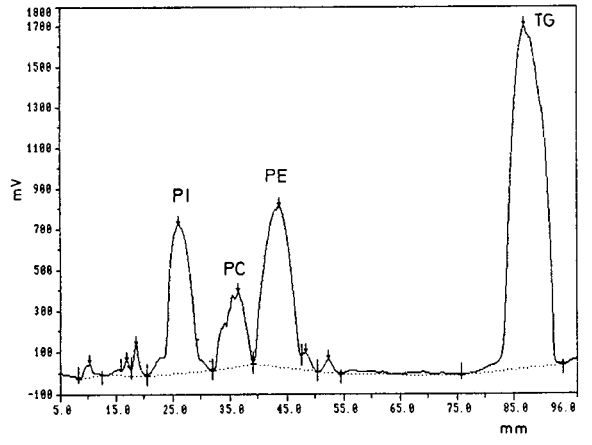

Fig. 2. Densitogram of a standard mixture (PI, PE, PD and TG) developed with optimized system: chloroform–methanol–1-butanol–25% (w/v) aqueous ammonia–ethyl acetate–0.25% (w/v) Ca^{2+} (80.2:44.1:4.9:5:5:6, v/v). (Ref. ref. 19, with permission).

4. PREPARATIVE TLC

Ready-to-use preparative TLC plates (20 × 20 cm, layer thickness 0.5 mm) have loading capacities (for, *e.g.*, crude lecithins) of *ca.* 30–35 mg, when applied as a band. This generally allows enough material, such as PE and PC, to be recovered for further analytical evaluation in a single TLC run.

4.1. Argentation chromatography

Ruminant milk fat consists of a complex mixture of TG molecular species, composed of fatty acids, ranging from C_2 to C_{24}, with odd-numbered, branched-chain and *cis–trans* isomers. This complexity presents a major analytical challenge.

Myher *et al.* [20] investigated a (25-year-old) molecular distillate of bobine butter oil (stored at 4 or −2°C), which was compared with a regular milk fat triacylglycerol fraction, enriched in C_4 and C_6 acids. The triacylglycerols of the distillate were purified by preparative TLC on silica gel. The TGs were then submitted to preparative argentation TLC (silica gel, impregnated with 15% $AgNO_3$), using chloroform as the developing solvent. The argentation TLC procedure resulted in seven bands, separated according to chain length, degree of unsaturation and the geometrical configuration of the double bonds. Each of the seven bands was recovered from the plate and submitted to polar-phase gas chromatography in conjunction with mass spectrometry (GC–MS). By utilizing GC–MS the molecular species pattern of a number of triacylglycerols in the TLC fractions was determined. The results clearly showed the complexity of the material and indicated that prefractionation was essential in order to obtain a reasonable resolution by GC.

It is possible that the food chemist would have benefitted more from the results of this investigation if it had been performed on a more readily obtainable material.

Argentation TLC was also used by McDonald *et al.* [21] to separate methyl esters of partially hydrogenated soybean oil. The aim was to identify *trans*-diene isomers formed during partial hardening of vegetable oils. In this instance, safflower oil was also studied.

Preparative TLC plates, coated with silica gel G (impregnated with 20% silver nitrate) were used to separate the methyl esters of the above-mentioned soybean oil. With chloroform as the developing solvent, six bands were recovered from the plates, of which each of the four most retained contained *trans*-diene isomers. The formation of *trans*-dienes during hydrogenation was confirmed by GC of the fatty acid methyl esters and by ^{13}C NMR spectrometry. It was established that over 20% *trans*-dienes were formed during the hardening procedure of soybean oil.

All papers that address the topic of *trans*-fatty acid analysis are of importance [22].

Kallio *et al.* [23] used preparative argentation TLC to fractionate the triacylglycerols of Baltic herring (*Clupea harengus membras*) flesh into eight bands, mainly according to degree of unsaturation. For this purpose, silica gel 60 G plates, impregnated with 8% $AgNO_3$ was used. The total lipid content of the flesh was 5.2% (w/w) and the proportion of neutral lipids thereof was 69.1%. The TGs comprised 61.5% of the neutral lipids. The intact TGs of the four most unsaturated TLC bands were scraped off the plate and subsequently analysed by capillary supercritical fluid chromatography (SFC) with FID. The acylcarbon numbers of the TGs were thus determined with reference to authentic TG standards. Also, identification of methyl ester derivatives of the triacylglycerol fatty acids was also performed by GC–MS.

4.2. Normal-phase applications

The lipid composition of fat globule membranes from butter milk and butter serum of Murrah buffalo and Red Dane and Sahiwal cross-breed cow milk was determined by Sharma *et al.* [24], utilizing TLC (silica gel G) for the class separation, iodine vapour for detection and gravimetric methods of evaluation. The fatty acid composition of total lipid was determined as methyl esters by GC. The lipid composition of the ruminant milk fat investigated is shown in Table 2. In this context it is of interest that animal milk sphingomyelins (SPH) contain remarkable levels of C22:0, C23:0 and C24:0 N-acylated fatty acids [25], which in bovine milk can be as high as 21–25% each [26]. Fatty acids from sphingomyelins are derivatized to methyl esters by acidic methanolysis.

The paper cited [24] unfortunately did not include a chromatogram showing the separation achieved. It is usually difficult to resolve phosphatidylinositol from sphingomyelin; the latter also often has a tendency to split into two peaks in normal-phase chromatography.

Preparative centrifugal accelerated thin-layer chromatography (CA-TLC), utilizing the Chromatotron, Model 7924T, was used by Bergheim *et al.* [27] to fractionate 300- and 500-mg (load limit) batches of crude soybean lecithin and egg yolk lecithin into lipid classes. The lipid class compositions were determined gravimetrically from the collected fractions ($n = 3$) and their fatty acid compositions were analysed as methyl ester derivatives by GC.

The egg yolk sample was reported to contain 14.2% PE and 66.8% PC by weight. A slower migrating fraction (fraction 5) than PC was identified as PI (3.6%). This was a surprising observation as PI generally is not present in such amounts in egg lecithin [28]; instead, sphingomyelin, which has similar retention characteristics to PI on silica adsorbents, occurs at that level [29]. The fatty acid composition of fraction 5 was not shown and it might be speculated that this is because the N-acyl group of SPH does not derivatize to fatty acid methyl esters by the alkali methanolysis procedure used. It was also surprising that no docosahexaenoic acid, 22:6 ($n - 3$), was detected in either PE or PC of the egg yolk lecithin. A typical level of this acid is 5%.

A *trans* isomer of oleic acid (18:1) was also suggested in the list of identified fatty acids of the soybean lecithin fractions. It might be speculated that this fatty acid is instead *cis*-vaccenic acid, 18:1 ($n - 7$), which is usually present in minute amounts (*ca.* 0.5–2%) along with the regular oleic acid, 18:1 ($n - 9$), in soybean phospholipids.

The isolated fractions were submitted to ^{1}H and ^{13}C NMR spectrometry, but unfortunately neither spectra nor tables were shown for these unexpected results.

Nevertheless, CA-TLC (also called "rotation planar chromatography") has potential in the analysis or fractionation of food lipids, mainly because it is a forced-flow method which to some extent combines TLC with the flow-controlling capabilities of HPLC [5].

TABLE 2

LIPID COMPOSITION OF FAT-GLOBULE MEMBRANES FROM BUTTER MILK AND BUTTER SERUM OF BUFFALO AND COW MILK (AFTER REF. 24)

Figures are means of eight observations ± standard error of the means. Percentage of unidentified lipid fractions (*e.g.*, squalene, hydrocarbons and glycolipids) is not included in the data.

Lipid component (% of total lipids)	Buffalo		Cow	
	Milk	Serum	Milk	Serum
Total NL	71.20 ± 2.68	70.92 ± 2.19	66.61 ± 1.76	72.49 ± 0.70
TG	50.44 ± 0.95	49.08 ± 1.93	45.33 ± 2.19	49.74 ± 2.29
DG	9.41 ± 0.63	9.09 ± 0.94	9.60 ± 1.20	9.22 ± 1.25
MG	2.34 ± 0.72	2.45 ± 0.33	2.72 ± 0.21	2.80 ± 0.57
FFA	4.49 ± 0.69	5.49 ± 0.49	4.67 ± 0.50	5.95 ± 0.37
Cholesterol	3.38 ± 0.16	3.40 ± 0.35	3.02 ± 0.53	3.32 ± 0.86
CE[a]	1.14 ± 0.51	1.39 ± 0.46	1.27 ± 0.32	1.32 ± 0.11
Total PL	24.68 ± 1.21	26.85 ± 1.98	28.71 ± 2.43	21.40 ± 1.52
PI	1.29 ± 0.16	1.28 ± 0.18	1.16 ± 0.06	1.48 ± 0.56
PS	3.64 ± 0.36	3.44 ± 0.64	2.99 ± 0.24	1.92 ± 0.05
SPH	4.70 ± 0.63	5.47 ± 0.12	5.87 ± 0.54	3.38 ± 0.30
PC	7.26 ± 0.24	7.52 ± 0.52	8.94 ± 0.36	6.25 ± 0.58
PE	7.79 ± 0.54	9.14 ± 0.78	9.75 ± 0.71	8.37 ± 0.90

[a] Cholesteryl esters.

A chemometric [16] strategy for the classification of lecithins according to process performance (in this case "good" or "bad" emulsifying capability) was developed by Kaufmann *et al.* [30]. Preparative TLC was utilized to fractionate the major phospholipid classes (PI, PC and PE) from commercial batches of soybean lecithins. Preparative TLC plates (silica gel 60) and a solvent system consisting of chloroform–methanol–2-propanol–0.25% aqueous KCl–ethyl acetate (30:9:25:6:18, v/v) was used to achieve the separation. The recovered phospholipid bands were submitted to GC, establishing the fatty acid compositions of the classes. Further, the overall lipid class composition of each batch of lecithin was determined by HPLC in conjunction with evaporative light-scattering detection. Utilizing principal components analysis (PCA), partial least-squares (PLS) discriminant analysis [31] and the analytical results, the authors were able to define a statistically valid class, the "good emulsifiers", and predict emulsifying performance for the lecithins. A lecithin can be "bad" in numerous ways but "good" only in one, namely when it works in its application. The method can be used for quality assurance purposes.

5. TLC–FID

TLC with FID is a method that permits a better detector linearity than densitometry in the absorbance mode. The technique in general has been thoroughly described by Ranny [32] and recently reviewed by Ackman *et al.* [33].

The advantages of total lipid hydrogenation prior to lipid class analysis was investigated by Shanta and Ackman [34]. Egg lipids, chromatographically purified fish oil and triacylglycerols of sea scallop (*Placopecten magellanicus*) together with other lipids were used to explore the benefits of this procedure. Samples were applied on Chromarods-SIII (Newman-Howells, Mid-Wales, UK) which were later scanned in a Iatroscan Mark III equipped with a flame-ionization detector.

A significant increase in the response for the hydrogenated lioid samples compared with their respective natural origin materials was accomplished in all instances investigated. The highest value was found for the fish oil sample, showing a response increase (*RI*) of 45%. Depending on the solvent system used, a variation in the *RI* value was registered (Fig. 3).

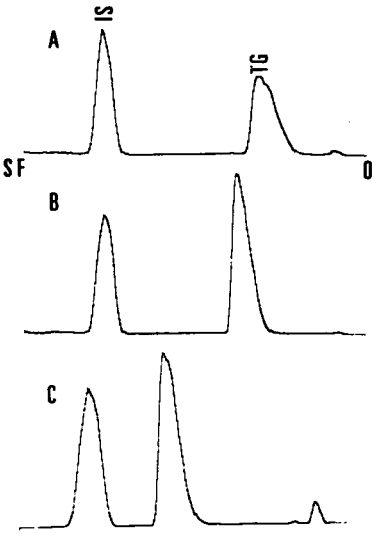

Fig. 3. Iatroscan TLD–FID showing the effect of hydrogenation on peak shape of fish oil TGs on Chromarods SIII. (A) Unhydrogenated. (B) Hydrogenated. Solvent system: hexane–diethyl ether–formic acid (97:3:1, v/v/v). (C) Unhydrogenated. Solvent system; hexane–chloroform (85:15, v/v), the chloroform containing 5% 2-propanol and 0.5% formic acid. IS = internal standard (wax ester); O = origin; SF = solvent front. (From ref. 34, with permission).

In general, the peak shape and the precision of peak areas were better for the rods spotted with hydrogenated lipids than for those containing the natural samples. This was explained by the fact that the number of different fatty acids was greatly reduced after completion of the hydrogenation process of the natural sample and hence the molecular species distribution narrowed down to only the saturates.

It was also interesting that hydrogenation decreased the resolution between PC and SPH, two classes which are structurally related (common polar head group, phosphocholine, and two lipophilic tails). This was due to the fact that the differences between the lipophilic tails of PC and SPH diminished on hydrogenation and hence separation was almost entirely achieved by the influence of the polar head group. Naturally, this was not beneficial to the separation.

A similar approach as that discussed above was successfully addressed by Ohshima and Ackman [35], utilizing hydrogenation of lipids prior to TLC–FID evaluation.

By modifying the construction of the flame ionization detector of the Iatroscan TH-10 Mark II instrument, its sensitivity, linearity and stability were improved [36]. This was done in order to monitor the phospholipid classes of Canola oil, the most widely used edible oil in Canada, during the various stages of oil processing. Przybylski and Eskin [36] adjusted the ion collector to 0.8 mm above the rods and installed a ball electrode, polarized as a detector cylinder inside the ion collector. As a result of these modifications, a tenfold increase in detector response was registered compared with the standard version of the Iatroscan analyser. It is believed that the improved response for phosphatidylserine (PS) and PI is due to better evaporation from the rod and improved combustion in the flame.

It is obvious that the performance of the Iatroscan analyser can be enhanced by technical modifications such as those described by Przybylski and Eskin [36]. Even though the improvement described above was excellent, there might be room left for further optimization of the detection system, possibly with the aid of chemometrics.

A number of factors (sample volatility, amount of substance, fatty acid composition, Chromarod movement rate through the flame, etc.) influence the response of different lipids in the flame ionization detector of an Iatroscan instrument. One way to partly solve these problems is to calibrate the system with standards identical with or similar to the analyte. This was the topic of a study conducted by Whitsett and Kennish [37].

The lipid class composition of Sockeye salmon muscle was determined by TLC–FID after calibration with standards similar to the fish sample in respect of the fatty acid composition. Results were presented for TGs, FFAs, PE and PC. An internal standard (pentacosane) was added to the natural samples which did not fully separate from the triacylglycerols on the Chromarods Type SII. Hence quantification was probably impaired. However, it was confirmed that the calibration sample should bear a close resemblance to the composition of the salmon muscle sample. The reader would also have benefitted from an indication of the variation of the method (for a natural salmon sample) rather than just the sample-to-sample variations listed in the paper.

A comparison between Kovacs *et al.*'s GC–FID

TABLE 3

CHOLESTEROL (INTERNAL STANDARD METHOD) IN SEAFOOD SAMPLES DETERMINED USING IATROSCAN TLC–FID (AFTER REF. 39)

Sample	Cholesterol (mg per 100 g)[a]
Atlantic cod (raw)	15.3 ± 2.40
Atlantic halibut (raw, frozen)	24.8 ± 2.30
Atlantic lobster meat (cooked)	83.6 ± 5.02
Atlantic mussels (cooked)	88.0 ± 12.07
Atlantic shrimp (boiled, shelled)	140.3 ± 19.6

[a] Mean ± S.D. (n = 7–10).

method [38] for the determination of total sterols in natural samples and an Iatroscan TLC–FID method was conducted by Walton *et al.* [39]. The total sterols of the edible portion of boiled shrimp and lobster, cooked mussels and skin free muscle tissue of fresh raw cod and frozen raw halibut, were determined by the two different methods.

The rods were developed in hexane–diethyl ether–formic acid (97:3:1, v/v/v) for 40–45 min, after which all sterols from the food samples appeared as a single peak on the TLC–FID scan, permitting the quantification of total sterol content. The use of an internal standard (5-α-cholestane), which was completely resolved from the total sterol peak, minimized the effect of rod-to-rod variations (cholesterol range 0–20 μg; r = 0.943). Utilizing calibration graphs, estimates of the cholesteral content of the seafood samples were made (Table 3). It was discouraging to find that lobster, mussel and shrimp delicacies are comparatively high-cholestrol hazards!

The TLC–FID method utilizing an internal standard for normalization, developed by Walton *et al.* [39], apparently produces quantitative results in agreement with a comparative GC standard method, hence it can be used for the screening and routine determination of sterols in foods.

6. CONCLUSIONS

A major reason for using planar chromatography for the analysis of food lipids is its flexibility (multi-dimensionality, multi-detectability, etc.) and the ease by which analytical separations are transferred to a preparative scale (*ca.* 30-mg loadings or 300–500-mg loadings in CA-TLC) to generate material for further analytical TLC evaluations, or by techniques other than TLC. One-dimensional TLC is a workhorse for comparative analysis, in quality assurance, process monitoring and quality control. It is the author's experience that the combined use of two different chromatographic techniques (predominately TLC and HPLC) greatly enhances the certainty by which retention time-based identifications can be made. Also, the sensitivity of TLC in lipid analysis (in conjunction with suitable detection reagents) is often better than that for the commonly employed evaporative light-scattering detectors used in HPLC.

Open-system chromatography is finding continuous new utilizations for a range of applications of which the analysis of food lipids does not appear to be in a particularly evolutionary phase. It is difficult to see any specific trend as to where researchers currently are focusing their efforts. On the whole, the advances in planar chromatography for the separation of food lipids has more the character of improvements to already existing techniques rather than breakthrough innovative work.

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Review

Thin-layer chromatography–flame ionization detection Iatroscan system

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ABSTRACT

The thin-layer chromatography–flame ionization detection (TLC–FID) Iatroscan system is a technique which is still being evolved. Quantification with the TLC–FID system relies heavily on the accurate setting up and calibration of the instrument. An appreciation of the factors that influence the analysis can eliminate significant errors. At least a few of the numerous operating variables need to be fixed to obtain coherent results from different laboratories. Hydrogenation of the sample is recommended in order to improve quantification with the Iatroscan system. The improved reproducibility obtained with automatic sample spotters compared with manual spotting indicates that autosampling is highly advisable.

CONTENTS

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1. INTRODUCTION

The Iatroscan is an instrument that combines the resolution efficacy of thin-layer chromatography (TLC) with the possibility of quantification by flame ionization detection (FID). This instrument is becoming increasingly popular in the field of food science, where it is being used extensively for lipid analysis. When the instrument was first marketed, more than two decades ago, there were controversies regarding the acceptability of the results obtained [1,2]. The major drawback of the instrument was a lack of quantitative accuracy and reproducibility. Relative standard deviations (R.S.D.s) as high as 30% and 83% [3,4] have been observed, especially for low sample loads. Since then, a number of papers have been devoted solely to the means of understanding the full working of the instrument and to increase its applicability in quantitative analysis [5–9]. This resulted in a better understanding of the working of the instrument, improved instrument design, especially the detector (making it more sensitive and reproducible), newer and more uniform Chromarods (the TLC component), better techniques for sample application, etc. Several reviews [5,7–9], an exclusive book [6] and a symposium volume on Iatroscan [10] have further helped in building the image of this instrument.

However, in spite of these several reviews and a better understanding of the instrument, it is not unusual still to find statements such as "the application of this method in lipid metabolism research tends to be limited" [11]. Whereas in a review in 1990, quantification of samples spotted on Chromarods without being subjected to solvent development (discussed later) was considered as irrelevant [7], in a more recent study such a technique was used for the determination of total lipids [12]. In two papers published in the same issue of a journal in 1991 there are contradictory statements regarding a particular aspect of the instrument (discussed later).

What is plaguing this instrument and why, even after two decades following its introduction, has it not found greater favour with analytical researchers and not yet been more widely adopted in quality control laboratories? With this background, it is not surprising that the TLC–FID system receives scant mention in *Chemical Abstracts* and *Analytical Abstracts*. This is because the users of this instrument generally tend to fall into two categories: those who routinely use it for quantification purposes and find it satisfactory, and others who have certain doubts regarding the acceptability of the results obtained. In an effort to make this instrument appear acceptable, most papers and reviews tend to overlook its drawbacks.

One of the disadvantages with this instrument is that it has several operating and user variables, only a few of which have been fixed by the manufacturers. A change in even one of the variables affects the results, which, in part, accounts for the conflicting observations from different laboratories. This necessitates a thorough standardization of the instrument and the procedural work, and, most of all, an understanding of the working of the instrument.

In this review some emphasis will be placed on understanding the instrument. The various aspects of which one needs to be aware when using the instrument will be treated in an uncomplicated and simple manner. The literature comparing TLC–FID with standard instrumental techniques will be covered and some applications of TLC–FID to foods, with special reference to the oil and fat industry, will be discussed.

2. INSTRUMENTATION

The Chromarod Iatroscan system consists of two independent units, the Chromarods which constitute the TLC component and the Iatroscan, the FID scanner unit. An early review by Ackman [5] and an excellent book on the TLC–FID Iatroscan system

[6] give a thorough illustration of the instrument. Therefore, only a brief discussion of the instrument will be given here.

The Chromarod is a quartz rod with a diameter of 0.9 mm and a length of 152 mm. A 148-mm length of the rod is coated with a thin layer (75 μm) of a mixture of soft glass powder and the adsorbent, either silica gel (Chromarods S, SII and SIII) or alumina (Chromarod A). These differ in the nature of the absorbent or its particle size [7]. The recently introduced Chromarod SIII is the only format now available [13].

The Iatroscan FID scanner consists basically of a hydrogen flame jet and an ion collector. The sample is burnt, the ions are collected by the collector electrode and the signal is amplified in a similar way as in the gas chromatography (GC)–FID.

The newer models of the instrument, the Mark IV and Mark 5, have an improved detector [7,8]. The ion collector is in close proximity to the Chromarods, making it a more efficient ion collector. The modified detector in the newer model was illustrated schematically in an recent review [7]. The Mark 5 is an improvement on the Mark IV in terms of operational features, doubled amplification to improve the detection limits, a built-in air pump unit and a decreased instrument mass [14].

The validity or accuracy of the TLC–FID method is a question of using the most suitable response factor (correction factor) for the sample. Hence it will be useful to consider the various factors that could affect the response of the flame ionization detector of the Iatroscan system.

3. FACTORS THAT AFFECT THE FID RESPONSE OF THE SAMPLE IN THE IATROSCAN SYSTEM

3.1. Chromarods

Chromarods are the heart of the TLC–FID Iatroscan system. The Chromarods come in a set of ten that can be mounted on a frame. The FID responses are shown to vary between the different sets of Chromarods and also from rod to rod within a set. Its has been suggested that one should match and select ten Chromarods having similar characteristics from a larger batch, in order to avoid rod to rod variation [7]. The grouped set of ten rods should then be treated in a similar fashion. Although this practice is desirable, it is expensive [a set of ten Chromarods SIII cost *ca.* US $215 (RSS, CA, USA)] to buy large numbers of Chromarods and moreover the procedure is time consuming. In such instances it is more advisable to consider each rod as a single isolated analytical unit and construct a calibration graph for each rod [7]. This may appear to be tedious but is a definite advantage as results can then be expressed with more confidence without any problem of rod to rod variation. The newly introduced SIII rods, being machine made, are more uniform. Sebedio and Juaneda [15] contend that the rod to rod variation among the SIII rods is low and hence it is not necessary to treat each SIII rod as a entity. However, it is essential to test a newly opened set of rods before assuming any such uniformity.

3.2. Nature of the sample

The response of a substance in TLC–FID is strongly affected by its composition. Hydrocarbons have the highest ionization abilities, whereas compounds containing heteroatoms such as oxygen, phosphorus or halogens give a lower response. Sterols are reported to give a high response, probably owing to the planarity of the molecule [7]. Even within the same lipid class the response varies depending on the molecular complexity of the substance. Excellent examples are natural samples such as fish oil triacylglycerols (TAGs), which give a much lower response than an equivalent mass of pure standard triolein. The multiplicity of the fish oil TAGs results in partial subfractionation on the efficient Chromarods. This results in shoulders or broadened peaks which give a lower response. Fraser and Taggart [16] observed a 1.7 times higher response for triolein compared with an equivalent amount of fish oil (Marinol) triacylglycerols.

The various suggestions in the literature concerning this discrepancy include the following: (a) if the peak is too broad, it will be advantageous to use a slightly more polar solvent; the subfractionation effects are not as marked in a polar solvent, hence a narrower peak could be obtained (Fig. 1) [13]; (b) double development in the same or a different solvent system can sometimes improve the FID response by producing a narrower band on the Chromarod [3]; (c) the use of reference standards more

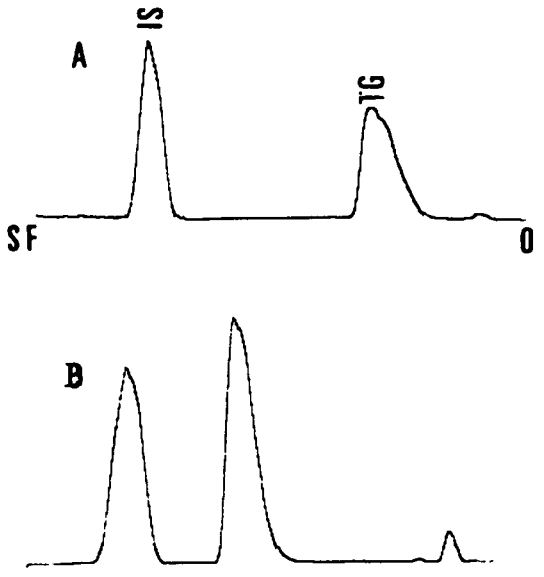

Fig. 1. TLC–FID showing the effect of the developing solvent on the peak shape of fish oil triacylglycerol on Chromarods SIII. (A) Developed in solvent system hexane–diethyl ether–formic acid (97:3:1, v/v/v); (B) developed in solvent system hexane–chloroform (85:15, v/v); the chloroform contains 5% of 2-propanol and 0.5% of formic acid. IS, internal standard, wax ester; TG, triacylglycerols; O, origin; and SF, solvent front. From Shantha and Ackman [13].

closely resembling the composition of the substance being analysed for TLC–FID calibration purposes [16]; purified natural standards or, when not available, synthetic standards could serve the purpose, but such standards can be expensive and unsaturated standards are less stable; (d) hydrogenation of the sample (discussed later) also produces an increase in the response by removing subfractionation effects due to the presence of an unsaturated system [13,17–19]

3.3. Amount of sample spotted and linearity of response

The response factor of any lipid class depends on the amount of sample spotted. The non-linearity of the FID response in the TLC–FID system even within a small working range comes as a shock to those accustomed to the FID response in GC. The suitable working range of the sample size is one in which the FID response is linearly dependent on the sample amount. Most calibration graphs of FID response *versus* sample mass obtained with the earlier instruments, equipped with the older detector, extrapolated to negative intercepts on the ordinate, making it unsuitable for low concentrations (<1 μg). In such cases of very low sample concentrations, as frequently encountered in aquatic samples, multi-level extensive calibration graphs at such low sample loads need to be constructed before the results can be considered acceptable [3].

The later Mark IV and Mark 5 models, with an improved detector design, are claimed to give good linearity over the working range up to 25 μg, with as little as 2.5 ng giving a measurable response [7]. However, in order to obtain a reasonable signal-to-noise ratio, it is recommended that sample amounts should exceed 10 ng [14]. Fig. 2 gives the calibration graphs for different lipid classes taken in the ranges 20–10 240 and 20–640 ng [15]. The Mark IV model, with an improved detector and the new Chromarods SIII, were used in this study. Note that the graphs are not linear over the range studied and that the response of the triglyceride (trilinolein) is different from that of cholesteryl ester.

Fig. 3 gives calibration graphs for three lipid classes [8]. The samples were developed on Chromarods SIII and the Mark IV model was used for scan-

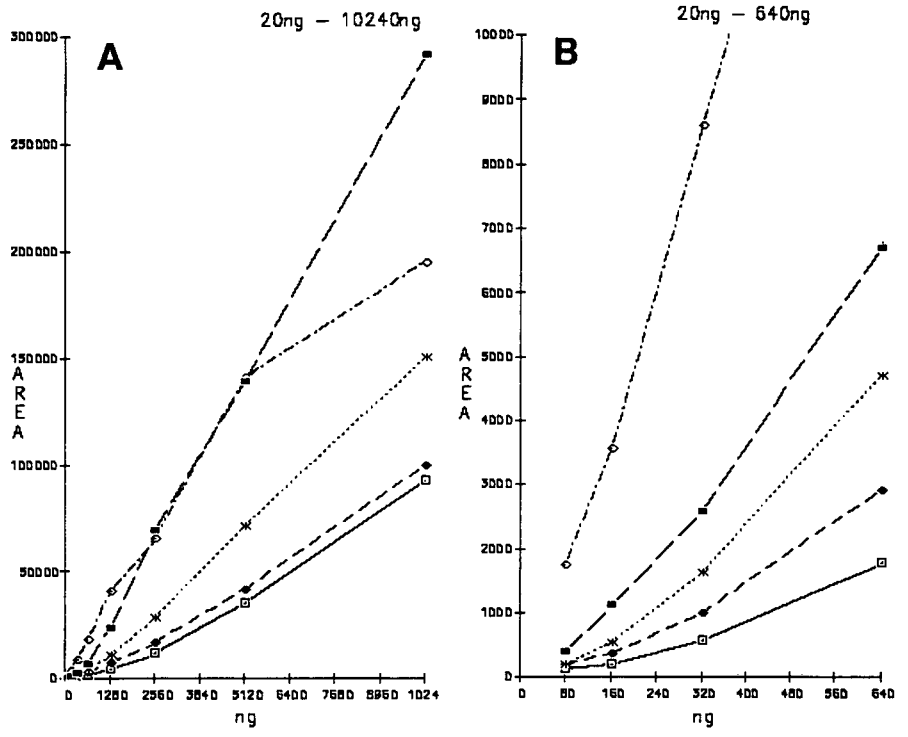

Fig. 2. Mass area response for (□) trilinolein, (◆) linoleic acid, (×) cholesterol, (○) cholesterol ester and (■) phospholipids developed on Chromarods SIII and scanned in the later model of the Mark IV Iatroscan. (A) Sample mass from 20 to 10 240 ng; (B) mass from 20 to 640 ng. From Sebedio and Juaneda [15].

ning. The graph is linear, the response of cholesteryl ester and triglyceride (species not shown) having similar responses. Of course, an exact comparison of Figs. 2 and 3 cannot be made, as little of the operating history is known. This is just an example of where two laboratories differ regarding the linearity of the response obtained from the newer improved detector of the Mark IV instrument and using the more uniform Chromarods SIII. Both studies [8,15] agree that the sensitivity and reproducibility have been improved with the later Mark IV model.

3.4. Sample preparation, spotting technique and conditioning of Chromarods

The sample needs to be dissolved in a suitable solvent before application on the Chromarods. Chloroform–methanol (5:1 or 2:1, v/v) is suitable for the dissolution and application of lipid samples on the Chromarods.

The spotting technique is a major factor that effects quantification with the Iatroscan. A large drop spotted on the Chromarod would spread on the rod, the spreading being more pronounced with alumina than silica rods [6]. This would result in a broadened band with a lower FID response. A sufficiently concentrated solution can be applied in a single aliquot of up to 0.5 μl. With dilute samples where relatively large volumes (10 μl) need to be spotted, it should be spotted in repeated aliquots allowing for drying of the solvent between each application. In such instances a drying lamp or a hotplate can be used to dry the solvent. In any event, the spreading of the solvent is inevitable when spotting such large volumes. Solvent focusing [3,20], *i.e.*, developing the rods in a polar solvent, such as acetone, to just above the point of application would refocus the spread out solute into a narrow band. A Drummond Microcap disposable pipette (1–10 μl) can be used for spotting; the use of a syringe fitted with a repeating dispenser or an automatic applica-

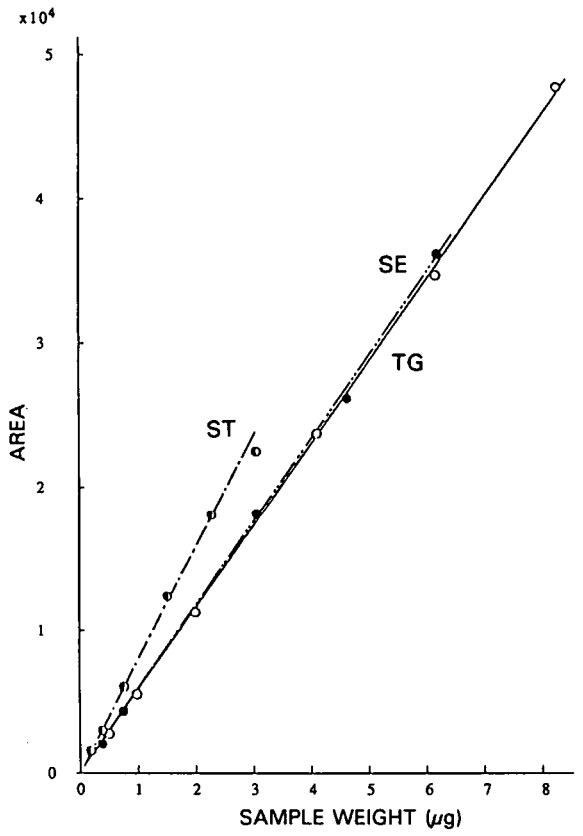

Fig. 3. Mass area response for three neutral lipid derivatives in hexane–diethyl ether (6:1, v/v) on Chromarods SIII and scanned in the later model of the Mark IV Iatroscan. TG, triacylglycerols; ST, cholesterol; SE, cholesteryl ester. From Iatron Laboratories, Tokyo, and Ohshima and Ackman [8].

tor is more suitable. Read [21] compared the different spotting techniques and suggested an improved rotary sample application method for the Iatroscan. In a recent paper, Sebedio and Juaneda [15] report a better R.S.D. (6.6%) for samples spotted using a autospotter than for manually spotted samples (10.1%).

The activity of the adsorbent on the Chromarods is strongly effected by the relative humidity of the laboratory atmosphere. The rods absorb moisture at a high rate, producing drastic changes in the chromatographic behavior of the rod. The few minutes during which the rod is exposed to the laboratory atmosphere during spotting could produce a decrease in the reproducibility of separation and response. It is recommended that the rods be dried in a desiccator for 5 min to remove absorbed moisture prior to developing in the solvent [7]. Alternatively, they can be conditioned at constant humidity (*e.g.*, 30%) by placing them in a closed chamber containing saturated salt solution [3,7]. The main aim of these treatments is to make the Chromarods have similar properties that can be easily reproduced during experiments, thereby allowing more consistent results.

3.5. Developing solvent and relative position of the sample on the Chromarod

The response of any lipid class is affected by the distribution of the sample along the Chromarod (Fig. 4) [3,7,22]. The sample material spotted on the Chromarod and subjected to development in a solvent is affected by the extent of its exposure to the solvent. A spot located nearer to the origin, which has solvent constantly passing through it, gives a

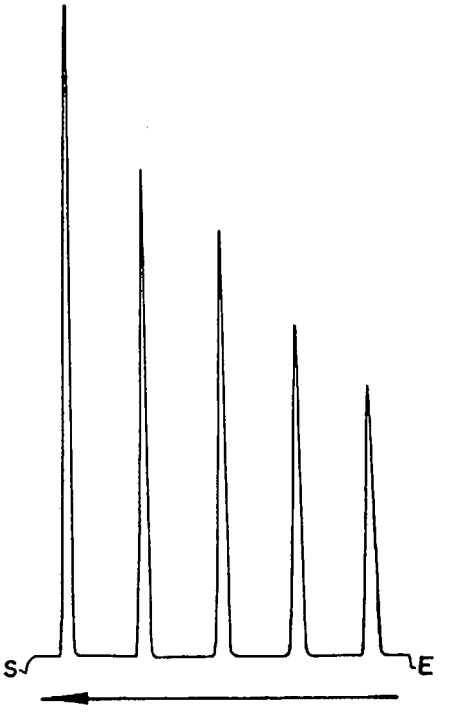

Fig. 4. TLC–FID showing the relationship between the distribution of the material (hexadecanone) on the Chromarod and the peak shape, and thus the response. The rod was developed for 40 min in hexane; S and E denote the start and end of the scan. From Parrish and Ackman [22].

short and broad peak having a low response. Variations in response could also arise from the non-uniformity of the adsorbent layer on the rods, the rods not being isotropic along their length. Kramer *et al.* [12] recently observed a significant positional effect in the FID response of methyl heptadecanoate spotted at nine different positions on the Chromarod, which was not subjected to any solvent development. This suggests that the positional variations are not necessarily due to solvent exposure, but could also be due to the anisotropy of the Chromarod. The newer Chromarods SIII, which are machine made with a more uniform adsorbent layer, need to be assessed for this effect.

In a few studies the sample material was analysed by FID without subjecting it to development in any solvent. Although this is known to result in split peaks, Kramer *et al.* [12] did not observe any such peak splitting of an undeveloped sample. They observed a large decrease (about 46%) in the FID response for the same amount of lipid sample on the same rods following development. This necessitates that the two techniques of analysis, *i.e.*, analysis of sample spotted on Chromarods that have been subjected to solvent development and analysis without subjecting them to any such development, be considered as separate studies. The solvent chamber has to be saturated with the solvent before inserting the rods for development. The eluent system and developing time (as the solvent front is not very visible) should be strictly adhered to in order to obtain reproducible results. The developing time depends on the solvent used and needs to be standardized in each laboratory. The temperature at which the rods are developed should also be maintained constant, as this has been shown to affect the response by producing a change in the R_F values [3]. A wealth of literature is available on the solvent systems to be used to effect the separation of a variety of samples [5–7] and details will not be repeated here.

3.6. The FID unit

3.6.1. Gases

The response of the detector varies with the flow-rate of hydrogen, although it is not as drastically affected by the flow-rate of air. Flow-rates of 2000 ml/min for air and 160 ml/min for hydrogen are generally recommended [5]. However, many studies show that a higher rate of 173 ml/min [3] or 180 ml/min of hydrogen gives better response and reproducibility [7]. Hence, depending on the nature of the sample, the flow-rate of the FID gases can be adjusted to obtain the optimum response and better reproducibility. Caution is needed in increasing the hydrogen flow-rate, as extreme heat may damage the frit of the Chromarod and shorten its life. High purity of the FID gases is a prerequisite for minimizing the baseline noises.

3.6.2. Scan speed

The response of the FID is dependent on the rate at which the rods pass through the flame, *i.e.*, the scan speed. In later models, the instrument is provided with a speed selector with numbers ranging from 1 to 5 and with scan speeds ranging from 2.5 to 5.1 mm/s. The recommended scan speed should allow complete combustion of all components while still being fast enough to avoid unnecessary thermal damage of the Chromarods [5,6].

3.6.3. Configuration of the detector

The response and reproducibility vary with the position of the Chromarods with respect to the flame and the ion collector. Przybyiski and Eskin [23] have modified the existing detector of the Mark II instrument to improve its sensitivity, linearity of response and stability. They adjusted the ion collector closer to the Chromarods (0.8 mm above the rods) and installed a ball electrode inside the ion collector. Kramer *et al.* [24] observed a fourfold increase in response when the ion collector was adjusted 1.7 mm above the rods rather than with the recommended space. In the newer models (the later Mark IV and Mark 5) the ion collector is in close proximity to the Chromarods, making it a more efficient ion collector. The detector is said to be more sensitive, with detection limits as low as 2.5 ng, and to give better reproducibility [8]. The linearity of response over a wide sample range, however, needs to be studied further. It will be useful if users of the newer model can provide information on the reproducibility and linearity of response that can be obtained with the instrument. The introduction of the commercially available flame thermionic ionization detector (FTID), which can be fitted on the older models, is useful for specific detection of nitrogen and halogen compounds [25,26].

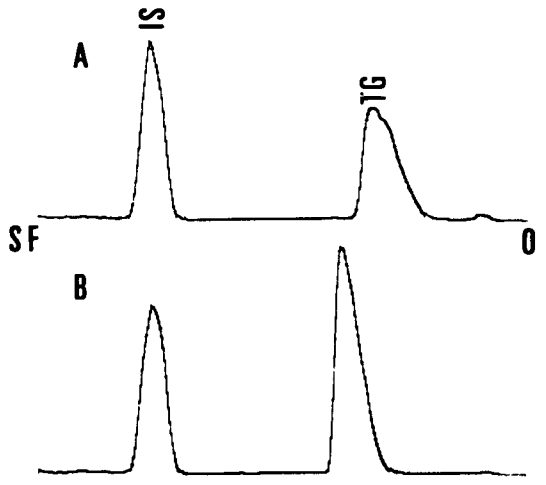

Fig. 5. TLC–FID showing the effect of hydrogenation on the peak shape of fish oil triacylglycerols (TG) on Chromarods SIII. (A) Unhydrogenated; (B) hydrogenated, developed in solvent system hexane–diethyl ether–formic acid (97:3:1, v/v/v). IS = internal standard; SF = solvent front. From Shantha and Ackman [13].

4. TECHNIQUES DEVELOPED TO IMPROVE ANALYSIS BY TLC–FID

4.1. Hydrogenation

Hydrogenation of unsaturated samples prior to TLC–FID has been shown to produce an increase in the FID response [13,17–19]. Hydrogenation can be performed in the laboratory by passing hydrogen through the sample in a suitable solvent and in the presence of platinum oxide (Adam's catalyst) with constant stirring. A detailed description of the hydrogenation procedure is given elsewhere [13]. The advantages of hydrogenation include (a) greater stability of the sample, thereby allowing for its analysis at a later time; (b) an important and useful increase in response for most lipid classes, including those containing only one double bond (the increase in response following hydrogenation was as high as 45% in the case of fish oil triacylglycerols [13]); (c) better peak shapes, as the subfractionation effects due to the presence of double bonds is nullified following hydrogenation (Fig. 5) [13]; (d) a better separation of lipid components in most instances, with a few exceptions (Fig. 6) [13]; (e) a lower R.S.D., *i.e.*, better precision, than for unhydrogenated samples; and (f) a simpler choice, lower cost and higher stability of standards that need to be used as references.

Exposure of Chromarods to iodine after the development of a sample was shown to increase the response [27]. Although this tends to saturate the double bonds by the addition of iodine, it is not as advantageous as hydrogenation and has not found many applications.

4.2. Impregnation of the Chromarods

4.2.1. Copper(II) sulfate impregnation

The response of a lipid class is higher on the impregnated rods than on unimpregnated rods [14,28–30]. Copper(II) sulfate could bring about a more uniform ionization of the sample, which could be lost by simple volatilization/pyrolysis using unimpregnated rods. Moreover, the copper could react with the organic compound at high temperatures of the flame, giving an organometallic compound, with an increased response [28]. The various advantages of using copper(II) sulfate-impregnated Chromarods for analysis include (a) an increase and a more uniform response for most lipid classes, (b) better reproducibility, (c) minimization of rod to rod variations, (d) better visibility of the solvent front and (e) improved baseline stability. The resolution of phospholipids is said to fall following impregnation of the Chromarods with copper(II) sulfate [30,31]. Hence a change in the composition of the developing solvent from that used when using unimpregnated rods is necessary to obtain optimum resolution.

4.2.2. Boric acid impregnation

Impregnation of Chromarods SII with 3% boric acid solution has been used for the complete separation of glyceride hydrolysis mixture (triolein, 1,3- and 1,2-diolein, 1-monoolein and oleic acid) [32]. Boric acid impregnation has been used to effect resolution between mono- and diglycerides and isomeric polyhydroxy fatty acids [33]. The principle of this separation is the same as that in silica gel TLC.

4.2.3. Silver nitrate impregnation

Impregnation of Chromarods with silver nitrate has been used to separate and determine geometric and positional isomers of fatty acids and different

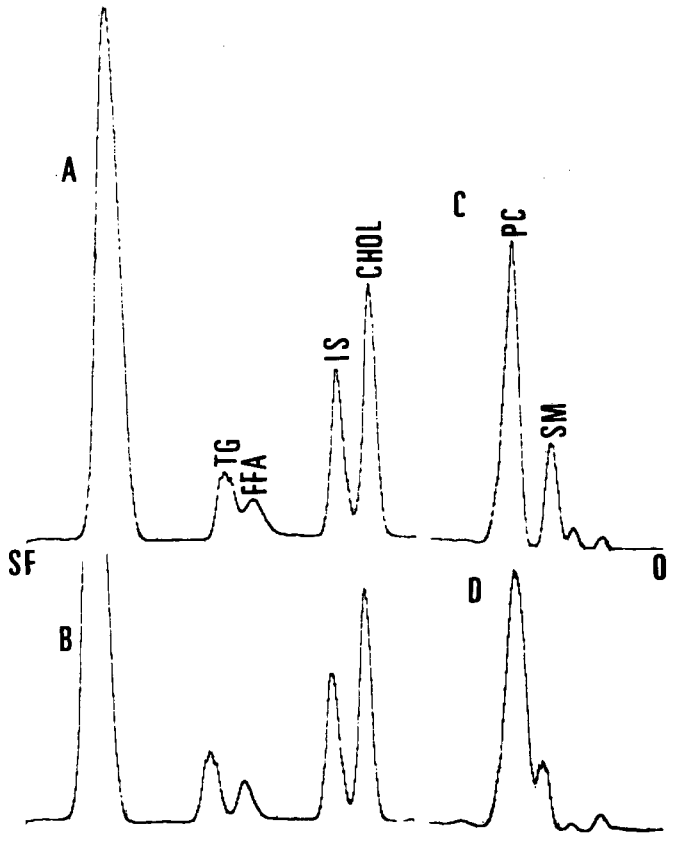

Fig. 6. TLC–FID showing the effect of hydrogenation on the peak shape and separation of human plasma lipids on Chromarods SIII in solvent system hexane–diethyl ether–formic acid (90:10:1, v/v/v), partial scan followed by complete redevelopment for analysis of polar lipids in solvent system chloroform–methanol–water (70:30:3.5, v/v/v). (A) Neutral lipids, unhydrogenated; (B) neutral lipids, hydrogenated; (C) polar lipids, unhydrogenated; (D) polar lipids, hydrogenated. SE = steryl esters; FFA = free fatty acids; IS = internal standard, fatty alcohol; SM = sphingomyelin; PC = phosphatidylcholine; TG = triacylglycerol; O = origin and SF = solvent front. From Shantha and Ackman [13].

species of triacylglycerols [34–36]. The rods need to be freshly impregnated before each analysis.

4.2.4. Oxalic acid impregnation

Oxalic acid impregnation of Chromarods is used to give an improved separation of phospholipids [37,38]. Impregnation with oxalic acid resulted in improved peak shapes and gave a good separation between phosphatidylinositol and phosphatidylserine. The oxalic acid concentration in acetone was found to be critical; concentrations lower or higher than 0.01 *M* were not satisfactory [38]. The Chromarods need to be impregnated each time before use.

A general note to add here is that with the newer type of instrument and more efficient Chromarods now available, impregnation of the rods should be done only if absolutely essential. When using these impregnation techniques the question arises of how often the impregnated Chromarods can be used before they need to be impregnated again. Silver nitrate-impregnated Chromarods can be used only once. Boric acid-impregnated rods have been used five times before renewing the impregnation [33]. Copper(II) sulfate-impregnated rods can be used for up to 25–30 scans [28]; more recently, Ranny *et al.* [31] observed that the rods need to be impregnated every few analyses, at least for the analysis of acidic phospholipids. The phospholipids seemed to react with the copper(II) sulfate. A white spot was

observed on a brown Chromarod at the site where phospholipid was present, after combustion [28]. Silver nitrate impregnation of the Chromarods is said to decrease their lifetime [6].

5. COMPARISON OF TLC–FID WITH CONVENTIONAL METHODS

From time to time a number of studies have been carried out using TLC–FID for various analyses and the results compared with those obtained with conventional methods. As no review has specifically covered this topic, it is hoped that this section will effectively provide information as to whether one can substitute an often time-consuming and elaborate conventional method with the rapid TLC–FID Iatroscan method.

5.1. TLC–FID Iatroscan vs. spectrophotometry for determination of phosphate value

Table 1 gives representative figures taken from two different studies [23,39]. In study 1 [39], the phospholipids (PLs) of oil were separated by column chromatography. The PLs were then applied to the Chromarods SII and developed in chloroform–methanol–water-based solvent system and scanned in an Iatroscan Mark III. The phosphate content was calculated as μg/g oil for individual PL peaks using a modified equation [39]. The phosphate content of each PL was then pooled to give a total phosphorus content. As a reference method the phosphate content was measured by means of the ash–spectrometric method [40]. In study 2 [23], the PLs were separated by two-dimensional TLC and analysed in a similar manner as in study 1 by TLC–FID. However, the detector of the Iatroscan Mark II was modified to give better sensitivity, stability and linearity of response [23]. Copper(II)-impregnated Chromarods were used. The ash–spectrophotometric method was used as the reference method. It is apparent that in both studies there is fair agreement between the phosphate value obtained using both the TLC–FID and the spectrophotometric methods, although the former gives slightly lower results. The higher values obtained by the spectrophotometric method has been suggested to be due to the presence of non-phospholipid phosphorus such as inorganic phosphates.

TABLE 1

COMPARISON OF TLC–FID AND SPECTROPHOTOMETRIC METHODS FOR THE DETERMINATION OF PHOSPHORUS CONTENT IN PROCESSED OILS

Plant oil sample	Phosphorus content (ppm)	
	Iatroscan	Spectrophotometry
Canola oil[a]		
Solvent	489 ± 5	506 ± 5
Expeller	172 ± 3	184 ± 3
Degummed	40 ± 2	42 ± 2
Corn oil[b]		
Crude	245	296
Degummed	4.9	9.3
Refined	0	2.5
Peanut oil[b]		
Crude	536	543
Degummed	3.1	35
Bleached	2.1	24
Refined	0	27

[a] Adapted from Przybyiski and Eskin [23].
[b] Adapted from DuPlessis and Pretorius [39].

Other comparative studies include the PL compositions of trout gills [41] and rat heart [24]. Hazel [41] concluded that the results obtained by TLC–FID compared well (within 5%) with the traditional phosphate analysis for all PLs except phosphatidylinositol, where the values obtained from Iatroscan method were significantly higher (about 55%). No reason was given for this observation, however. Kramer *et al.* [24] studied rat heart lipids and concluded that the results obtained by the TLC–FID and the spectrophotometric methods for phosphate analysis were comparable, the standard error in the Iatroscan method, being higher, however, owing to wide variations in the results obtained.

5.2. TLC–FID Iatroscan vs. GC

5.2.1. Lipid analysis

Ranny *et al.* [42] compared the determination of commercial molecularly distilled acylglycerols by GC and TLC–FID methods. The results were found to be comparable with roughly the same reproducibility. The TLC–FID method was found to be preferable for serial analysis in process control or technical quality control. In a comparison of the

reproducibility of results obtained by the two techniques for the analysis of blood lipids, Mares *et al.* [43] concluded that the variations obtained in the Iatroscan analysis were much higher than those obtained by GC and that the former method needs to be improved to give better reproducibility. Rao *et al.* [44] evaluated the use of the TLC–FID system to study the lipid composition of alcohol-induced rat fatty liver. In most instances the values obtained by TLC–FID compared well with those given by GC, but in one instance the value obtained by TLC–FID was almost double that obtained by GC.

Sebedio and Ackman [34] analyzed a synthetic mixture of methyl stearate, oleate, linoleate and linolenate on silver nitrate-impregnated Chromarods and compared the results with those obtained from GC analysis using an open-tubular Silar 7CP column. Comparable results were obtained by the two methods for all methyl esters except methyl linolenate, the amount of linolenate measured by TLC–FID being considerably higher (18.7 ± 1.4%) than obtained by GC (11.8 ± 0.1%) [34]. Regarding precision, four GC analyses gave more reproducible results than ten Iatroscan analyses.

Beaumelle and Vial [45] transesterified crude lipid extract of human erythrocyte using boron trifluoride–methanol and determined the resulting FAMEs, dimethylacetal and total cholesterol by both GC and TLC–FID. The results correlated well, with TLC–FID giving lower precision than GC. They did not account for cholesterol methyl ether and cholestadiene, which are formed when cholesterol is reacted with boron trifluoride–methanol.

Table 2 gives representative figures taken from two different studies [46,47], wherein the sterol content obtained from GC and TLC–FID analyses are compared. Walton *et al.* [46] determined cholesterol in seafoods by TLC–FID and Kovac *et al.*'s GC method [48]. The unsaponifiables were spotted on Chromarods SII and developed with hexane–chloroform–formic acid. The results obtained with the Iatroscan with the use of cholestane as internal standard, without an internal standard and by GC were comparable. More recently O'Keefe [47] determined the sterols in caviar on alumina rods and compared the results with those obtained using Chromarods SIII and GC. The results from GC compared better with the alumina rods than silica when used without any correction factors [47]. It should be mentioned that GC is capable of resolving the different sterols, which is not the case with TLC–FID, hence only the determination of total sterols is possible by TLC–FID.

Bascoul *et al.* [49] compared the levels of polar oxysterols as measured by TLC–FID and GLC. The values obtained by TLC–FID analysis (341 ± 65 ppm) were considerably higher than those ob-

TABLE 2

COMPARISON OF TLC–FID METHOD (USING CHROMARODS SIII AND A) WITH GC FOR THE DETERMINATION OF CHOLESTEROL IN FISH PRODUCTS

Sample	Cholesterol content (mg per 100 g)		
	TLC–FID		GC
Atlantic cod (raw)[a]	15.27 ± 2.40		17.95 ± 2.70
Atlantic halibut (raw, frozen)[a]	24.77 ± 2.30		26.24 ± 2.61
Atlantic lobster meat (cooked)[a]	83.59 ± 5.02		101.12 ± 8.98
	Chromarods SIII	Chromarods A	
Caviar[b]			
Beluga	422 ± 43	378 ± 13	342 ± 47
Oestra	579 ± 29	455 ± 20	404 ± 36
Lumpfish	516 ± 67	469 ± 10	303 ± 28

[a] Adapted from Walton *et al.* [46].
[b] Adapted from O'Keefe [47].

tained by GC (124 ± 50 ppm). The authors are of the opinion that results obtained by TLC–FID were more correct as they agreed more closely with the cholesterol loss during heating (376 ± 37 ppm). However, GC is a better method for evaluating specific cholesterol autoxidation products owing to its better resolution capabilities.

5.2.2. Dimer acid components

Zeman *et al.* [50] and Fritz *et al.* [51] determined dimer acid components by TLC–FID and GC method. Both concluded that the results are comparable and that TLC–FID is a superior technique for dimerization process control owing to its speed and simplicity with no exhaustive sample preparation. Rao *et al.* [52] found the results obtained by Iatroscan to be comparable to those obtained by GC, and high-performance gel permeation chromatography for the determination of monomer, dimer and trimer contents in methyl esters of dimer acids.

The Iatroscan method was shown to give better precision than TLC–densitometry for the analysis of cosmetics [53]. Mills *et al.* [54], in an analysis of lipoproteins, were of the opinion that the results obtained using the Iatroscan were less accurate than those obtained by conventional methods.

The less accurate and less reproducible results obtained with the Iatroscan as compared with other methods could be due to the number of user and operating variables of the instrument. It could also be due, at least in part, to the lack of familiarity with this instrument. It can be envisioned that using an unfamiliar method would naturally lead to considerable variability in results as compared with an accustomed house method. The choice of a method in practice should be based on practicality, with the use of the least expensive and fastest method with the required degree of reliability.

At this juncture it is appropriate to pinpoint the advantages of the TLC–FID system over the conventional methods. The chief advantage of TLC–FID is the short analysis time, with the possibility of determining all components in a single analysis. The partial scanning and redevelopment [5–7] that can be done using the Chromarod Iatroscan system have no parallel in any other analytical technique. A good example of such a separation is illustrated in Fig. 7 [3]. As many as eleven classes of lipids were identified in marine samples using a combination of multiple developement schemes with the partial scanning facility of the Iatroscan. The ten Chromarods in a frame facilitate the analysis of ten samples at the same time. The high sensitivity of the improved detector permits the study of very small amounts (micrograms to nanograms) of sample. There is no need for elaborate sample preparation for TLC–FID analysis.

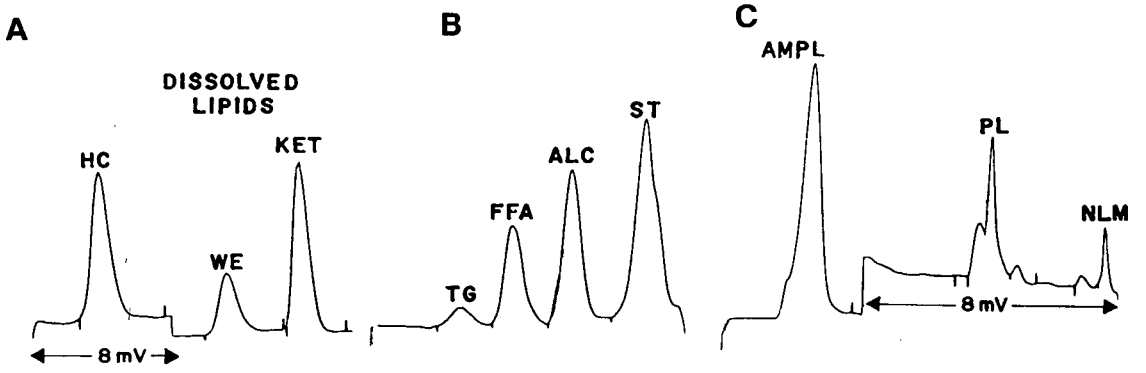

Fig. 7. Multi-class analysis of lipids using partial scan and redevelopment techniques. Each rod was scanned twice partially and once completely. (A) Double development in solvent system hexane–diethyl ether–formic acid (99:1:0.5, v/v/v), 25 min followed by 20 min, and scanned to the lowest point behind the ketone (KET) peak. (B) 40 min development in hexane–diethyl ether–formic acid (80:20:0.1, v/v/v) and scanned to the lowest point behind the sterol (ST) peak. (C) Last complete scan after double development first in acetone (15 min each development) and second in methylene chloride–methanol–water (5:4:1, v/v/v) developed for 10 min each. From Parrish [3]. Peaks: HC = aliphatic hydrocarbon, WE = wax ester, TG = triglyceride, FFA = free fatty acid, ALC = free aliphatic alcohol, AMPL = acetone-mobile polar lipids, PL = phospholipids, NLM = non-lipid material.

6. APPLICATIONS OF TLC–FID IATROSCAN SYSTEM

TLC–FID has widely been used in lipid analysis and this has been extensively reviewed [5–7] and will not be discussed further here. Only the few instances where TLC–FID has been applied to a specific problem in the oil and fat industry and which could be further developed to serve the food industry will be discussed.

6.1. Oils and fats analysis

TLC–FID finds wide application in the analysis of oils and fats [5–7]. Lipid contents in fish and deep-sea sediments have been determined using TLC–FID [20]. The sample, in chloroform–methanol (1:1, v/v), was spotted on the Chromarods. The entire sample was focused to one point by development in a polar solvent to just above the point of application. The sample was then scanned by the Iatroscan to give a total lipid measurement. A known standard having a composition similar to that of the sample was used as a reference to obtain accurate results [20]. More recently, Kramer *et al.* [12] determined the total lipids of *Methanobacterium thermoautotrophicum* using methyl ester as standard. The sample and standard were spotted alongside on the same copper(II) sulfate-impregnated Chromarod. The undeveloped rods were burnt as such in the flame ionization detector. The FID response factor of total lipids was assumed to be 1. The FID response of the standard was used to calculate the total lipid content [12].

TLC–FID has been used in industry for the analysis of castor oil [55]. The triacylglycerols (TGs) containing ricinoleic acid separate well from remaining TG species and can be easily determined. TLC–FID using silver nitrate-impregnated Chromarods has been used for the identification of various fats, such as beef tallow, olive oil and soya, cocoa butter, palm and coconut oils, rape, flax, corn and safflower oil [6]. TLC–FID with silver nitrate-impregnated Chromarods has also been used for determining *trans* fatty acids in margarine and partially hydrogenated oils and to study the methyl ester composition [34–36]. Kamata [56,57] determined the triglyceride composition of natural oils and fats such as lard, vegetable oils, fish oil and butterfat by the TLC–FID determination of their oxidation products obtained following their partial hydrogenation.

The presence of high levels of cholesterol in foodstuffs is of major health concern. Walton *et al.* [46] determined the cholesterol level in fish products using TLC–FID and O'Keefe [47] that in marine-based food products (caviar) using alumina rods.

6.2. Oil refining

The presence of phosphatides in refined oil could cause stability problems. TLC–FID has been used to study the effects of refining on the distribution of phospholipids (PLs) in vegetable oil. The acetone-insoluble compounds from crude and degummed rapeseed oil were precipitated and the total PLs were determined by TLC–FID using 1,2-dichloroethane–chloroform–acetic acid (94.5:1:1, v/v/v) as the developing solvent [6]. The immobile PLs remaining near the origin were determined. DuPlessis and Pretorius [39] studied the total and individual phospholipids in refined canola oil using TLC–FID and using an equation to convert the PL content into phosphorus content. The Iatroscan method was found to be accurate in determining the PL phosphorus in the range 145–536 ppm; however, for lower levels (1–10 ppm) of PLs phosphorus the method was not accurate.

More recently, Przybyiski and Eskin [23] made a similar study of canola oil using copper(II) sulfate-impregnated Chromarods and modified Iatroscan Mark II detector. The improved sensitivity of the detector and the higher response generally obtainable on the copper(II) sulfate-impregnated rods allowed the determination of PLs and phosphorus in the nanogram range [23]. In the oil refining industry the TLC–FID Iatroscan system can be put to good use to study the extent of free fatty acids present and the saponification process.

6.3. Quality assessment of oils and fats

The presence of polar artifacts in fats, which is often due to oxidized fats, could serve as a suitable index to assess their quality. The oxidized material, being more polar, does not migrate on the Chromarods when hexane–diethyl ether–formic acid is used as the developing solvent and thus can be easily determined. Kaitaranta and Ke [58] studied the ox-

idation of fish oils, such as mackerel, herring and tuna. The increase in the signal of polar lipids was found to be proportional to the increase in the mass of the sample, although no such correlation was found when compared with the thiobarbituric acid value. Ranny [6] determined oxidized and unoxidized methyl linoleate by TLC–FID. They observed a direct relationship between the area of the oxidized peak and the peroxide value. Katoh *et al.* [59] determined the oxidation products in edible oil (corn, safflower) and methyl linoleate by subjecting the sample applied on Chromarods to three developing solvent systems, followed by scanning in the Iatroscan analyser. Beef tallow heated under deep frying conditions has been evaluated for cholesterol oxidation products using TLC–FID [49]. The extent of monomer, dimer and polymer contents in oxidized fat can also be assessed by TLC–FID [52]. These various studies suggest that TLC–FID can be used to study the oxidative deterioration of fats and oils.

6.4. Production processes and process control

TLC–FID analysis has been used in routine production processes and process control applications. It has been applied to study lipase kinetics, glycerolysis and esterification reactions [6,7]. Ranny *et al.* [42] found TLC–FID to be useful to study distilled monoacylglycerols. Ackman *et al.* [60] used TLC–FID to study the time required for complete esterification of fish oil triacylglycerols using methanolic sodium hydroxide as the transesterification reagent. Zeman *et al.* [50] and Fritz *et al.* [51] applied TLC–FID to dimerization process control. In view of its simplicity and rapidity, with no exhaustive sample preparation steps, TLC–FID can be exploited to advantage in routine production processes.

Other applications of TLC–FID in the food industry include analyses of sugars, carbohydrates, amino acids, food additives such as emulsifiers, flavors, preservatives and antioxidants and food contaminants such as plasticizers; these have been discussed elsewhere [6].

7. CONCLUSIONS

Quantitative analysis using the TLC–FID system relies heavily on the accurate setting up and calibration of the instrument. An appreciation of the factors that influence the analysis can eliminate significant errors. One should remember that there are no experimental trivia in relation to Iatroscan analyses. It would greatly improve the image and acceptability of the TLC–FID Iatroscan system if the manufacturers in this field could come forward with models of Iatroscan instruments made in such a way that the entire detector can be replaced with a newer and more efficient detector. This would, at least in part, eliminate the cost of purchasing of a new instrument each time the detector is modified.

At least a few of the innumerable operating variables need to be fixed. For this, the positive points from various studies need to be taken into consideration, combined and assessed so as to give the best results. An inter-laboratory study using the best features could help in standardizing the technique for optimum results. From personal experience and from other studies, spotting is definitely one of the variables that needs to be standardized if reliable results are to be obtained. If the automatic sample spotter is as good as it is advertised to be, purchasers of the Iatroscan instrument should be encouraged to buy it. This may involve a higher initial cost, but would improve the accuracy and precision of the results by removing the large variations in results due to the spotting technique.

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Review

Gas chromatography of fatty acids

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ABSTRACT

Lipids in foods contain a wide variety of fatty acids differing in chain length, degree of unsaturation, position and configuration of double bonds and the presence of special functional groups. Modern capillary gas chromatography offers excellent separation of fatty acids. Fused-silica capillary columns with stationary phases of medium polarity and non-polar methylsilicone stationary phases successfully separate most of the natural fatty acids. Special applications, such as the separation of complex *cis–trans* fatty acid mixtures and cyclic fatty acids, require particular chromatographic conditions, including the use of very long capillary columns or more polar stationary phases. The derivatization methods for the preparation of fatty acid esters also need to be optimized to obtain accurate quantitative results. This paper reviews the derivatization techniques, capillary columns and stationary phases commonly used in the gas chromatography of fatty acids in food.

CONTENTS

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1. INTRODUCTION

The gas chromatography (GC) of fatty acid methyl esters with flame ionization detection (FID) is the most often used method for the analysis of fatty acids. The advent of wall-coated open-tubular (WCOT or capillary) chromatographic columns, now available with different stationary phases, offer excellent resolution capabilities regardless of the sample type. The differing nature of fatty acids present in various food groups, such as vegetable oils, including hydrogenated oils, dairy and meat fats and fish oils, requires different conditions in their GC analysis.

In this review, the various aspects of the transesterification of lipids, involving different reagents and derivatization techniques, are discussed. The performance of the most commonly used packed and capillary GC columns in the analysis of fatty acids in foods is evaluated.

The analysis of fatty acids involves mainly two steps: the preparation of the fatty acid methyl esters (FAMEs) and the analysis of the FAMEs by GC.

2. PREPARATION OF FATTY ACID METHYL ESTERS

It is necessary to optimize the derivatization step in order to obtain accurate quantitative results. In a study of the myocardial metabolism of fatty acids, by the GC analysis of FAMEs using boron trifluoride (BF_3)–methanol as esterification reagent, Al Makdessi *et al.* [1] concluded that the sample preparation procedure directly affected the quantitative results.

The common procedural deficiencies associated with ester preparation include (i) incomplete conversion of the lipids to FAMEs; (ii) alteration of the original fatty acid composition during esterification, including the formation of positional and/or geometric isomers; (iii) formation of artifacts which can be wrongly identified as fatty acids; and (iv) contamination and subsequent damage of the GC column resulting from traces of the esterification reagent (if not washed properly) or an unclean sample. Incomplete sample clean-up may result in the build-up of refractory material in the column and detector, which in turn will affect the resolution and quantitation.

Esterification reagents (referred here to reagents that convert free fatty acids to esters) and transesterification reagents (those which convert acylglycerols to fatty acid esters), generally fall into two categories: "acid-catalyzed" and "base-catalyzed" reagents. An early excellent review by Sheppard and Iverson [2] discussed in detail the various esterification and transesterification reagents. After a survey of the various reagents and methods, they concluded that "... there is no method of esterification that is ideal". They agreed, however, that most of these methods are acceptable if used properly. Bannon *et al.* [3] reviewed in detail base methoxide-catalyzed transesterification, which they referred to as methanolysis. They pointed out the various pitfalls and discrepancies associated with the published methods and proposed a suitable methodology taking into consideration the desirable features of the existing procedures.

2.1. Base-catalyzed reagents

Sodium methoxide in methanol is generally accepted for the conversion of the acylglycerols to methyl esters. Nevertheless, this technique is not very useful when large amounts of free fatty acids (FFAs) are present in the sample [4]. This transesterification method is rapid and can be carried out at room temperature. Under such mild conditions the reagent does not cause any isomerization of double bonds, nor does it liberate the aldehyde from the plasmalogens, which could complicate the chromatogram. The sphingolipids and cholesterol esters need more vigorous conditions for transesterification with sodium methoxide [4]. A disadvantage with this base-catalyzed transesterification reagent is that it does not convert the FFAs into FAMEs. The conditions need to be anhydrous as the presence of water causes saponification, thereby resulting in a loss of fatty acids. Negligent and prolonged use of this reagent may cause alterations in the fatty acid composition. Use of high concentrations of base and high temperatures could result in the formation of conjugated fatty acids.

Potassium hydroxide in methanol is another transesterification reagent [5] commonly employed in the transesterification of oils. Bannon *et al.* [3] discussed the superiority of the sodium methoxide over the potassium hydroxide in methanol method, taking into consideration that the latter could result in saponification. They found the methoxide–reflux

method (which takes only 2 min) to be suitable for the determination of fatty acids in a wide variety of fats. However, this technique is not accurate for the determination of short-chain fatty acid esters such as methyl butyrate and caproate, which are present in considerable amounts in dairy fat. The removal of the excess of sodium methoxide by washing with brine solution before GC analysis is strongly recommended, as the reagent tends to interfere in the analysis [3]. Sodium methoxide is a popular methylating agent in the oil industry, especially for refined oils, where the FFAs have already been removed during the refining stage [6]. A series of publications [3,6–9] from the Unilever group in Australia are devoted to alkali-catalyzed transesterifications and the accuracy of the GC analysis of fatty acids.

More recently, guanidine and its alkylated derivatives in methanol have been used to catalyze the esterification of oils and fats [10]. This basic reagent causes the complete methanolysis of oils, converting both the acylglycerols and the free fatty acids into methyl esters. The FFAs are said to form the salt guanidinium carboxylate, which in the presence of excess of methanol forms the methyl ester. Again, the excess of reagent needs to be extracted before the sample is injected into the GC column. Schuchardt and Lopes [10] compared guanidine-catalyzed methanolysis with the Ce 2-66 (20% BF_3–methanol) AOCS method [11] and the Hartman and Lago method (NH_4Cl–H_2SO_4 in methanol) [12] to study the fatty acid composition of several seed oils. The results with the first method were comparable to those obtained by the Ce 2-66 method, the linoleic acid concentration being slightly higher than that obtained by the Hartman and Lago method [12]. The reagent is inexpensive, and mild, causing no isomerization of double bonds. This derivatization reaction is rapid, requiring 2 min of heating in a boiling water-bath.

2.2. Acid-catalyzed reagents

Among the various acid-catalyzed reagents (such as methanolic hydrochloric acid, sulfuric acid in methanol and acetyl chloride in methanol), boron trifluoride in methanol has found wide application as a good reagent to convert both the acylglycerols and the FFAs into methyl esters. The popularity of this reagent may be due, in part, to its early acceptance in the American Oil Chemists' Society (AOCS) method [8]. The Association of Official Analytical Chemists (AOAC) procedure [13] involves the saponification of the acylglycerols using sodium hydroxide in methanol followed by esterification with BF_3–methanol to give the FAMEs. This method has been stated to be not useful for fatty acids containing unusual functional groups such as epoxy, cyclopropenyl, cyclopropyl, acetylenic or conjugated unsaturation. Although the first step is generally considered to be saponification, Ackman *et al.* [14] clarified that the first step, which involves the use of alcoholic sodium hydroxide, is a base-catalyzed transesterification and not saponification. Christie [4] is of the opinion that BF_3–methanol has been overrated as a transesterification reagent; it is known to form artifacts and is not particularly suitable for seed oils containing unusual fatty acids. The reagent reacts with the plasmalogens to liberate the aldehyde, which is then converted into dimethyl acetals. The dimethyl acetals (depending on the chain length) may interfere in the fatty acid analysis by GC [15]. The reagent reacts with cholesterol to give cholesteryl methyl ether and cholestadiene, which also interfere in the GC analysis of fatty acids. The reagent is expensive and has a limited shelf-life if not refrigerated. Use of old or concentrated reagents has been shown to result in loss of polyunsaturated fatty acids (PUFA) [4]. In spite of these various disadvantages, BF_3–methanol is currently accepted as being of great value for the rapid methylation of fatty acids.

Aluminum chloride in methanol has been used to transesterify a number of lipid samples [16]. With milk and vegetable oils the transesterification reagent was added directly without prior extraction of lipids. This reagent was comparable to BF_3–methanol for the transesterification of cholesteryl esters. The product of methylation obtained by using this reagent showed no artifact when analyzed by TLC, whereas the BF_3–methanol reagent showed some extra spots on the TLC plates. One of the disadvantages with the aluminium chloride reagent is that it does not esterify the FFAs. Hence it appears that in spite of being a Lewis acid, like boron trifluoride, it does not behave in a similar way.

2.3. Diazomethane

Diazomethane is a rapid esterification reagent

which esterifies FFAs to FAMEs in the presence of methanol [4]. The reaction is almost instantaneous at room temperature, the excess of reagent being easily eliminated by evaporation under nitrogen. However, diazomethane is extremely toxic. Moreover, diazomethane is known to form artifacts by reacting with double bonds or carbonyl groups [17]. The high reactivity of this reagent is said to work to its own limitations [17]. It is recommended that diazomethane be used only if absolutely necessary [4].

2.4. Other esterification reagents

The past decade has witnessed the emergence of a number of methods for the preparation of FAMEs. Most of the methods involved modification of existing procedures or incorporation of new reagents, and were invariably accented towards the rapidity of the method. With the large number of samples that need to be analyzed, there was a general tendency to seek rapid methods for esterification and analysis.

Strongly basic quaternary salts of ammonia such as *m*-trifluoromethylphenyltrimethylammonium hydroxide (TFMPTAH) in methanol [18], trimethylphenylammonium hydroxide (TMPAH) [19] and tetramethylammonium hydroxide (TMAH) [20–22] have found applications as transesterification catalysts for acylglycerols. The FFAs which may be present in the sample are converted into quaternary ammonium salts, which under the pyrolytic conditions of the gas chromatograph give the corresponding methyl esters. The advantages of these methods include a single-step transesterification, instead of the conventional saponification followed by esterification. These reagents do not require an extraction step, which is of particular concern when dealing with short-chain fatty acids. Metcalfe and Wang [20] have shown that with the use of TMAH as reagent, one can separately determine the fatty acid composition of the glycerides and the FFAs. With a slight modification the total fatty acid composition can be determined. However, the sample has to be neutralized before injection into the GC system. The high alkalinity and the relatively high column temperature in the pyrolytic methylation step often cause isomerization of the double bond, resulting in the formation of conjugated acids. The excess of TMAH breaks down to give trimethylamine, methanol and dimethyl ether with acceptable GC properties [23]. TMTFTAH on breakdown gives *m*-trifluorotolyldimethylamine, which has been shown to interfere in the separation of medium-chain fatty acids [23]. Trimethylsulfonium hydroxide (TMSH) is another single-step transesterification reagent that requires no additional extraction. The product of its pyrolysis, dimethyl sulfide, elutes with the solvent peak during GC analysis [23].

Emphasis has also been placed on a one-step digestion, extraction and esterification of lipid samples, which has been referred to as "direct transesterification" by most researchers [24,25]. This method circumvents the extraction and isolation of lipids, thus saving considerable amounts of time and chemicals. The sample is taken in a solvent and treated with acetyl chloride in methanol to give FAMEs. This method has been modified in that different solvents have been used. The transesterification reagent, heating time and temperature have also been modified. This direct method has been applied to determine the fatty acid composition of different tissues, plasma, feeds, faeces, milk fat, etc. [24–26]. Sukhija and Palmquist [26] carried out the experiment both in benzene and in chloroform. However, when using chloroform one should be careful because the ethanol present as a stabilizer in chloroform could result in the formation of ethyl esters, which would interfere in the GC of FAMEs [27]. Chloroform, in the presence of base and heat, forms dichlorocarbene, which reacts with the double bond of unsaturated fatty acids [4]. The hazardous benzene can be replaced with toluene.

The direct method has been compared with the traditional extraction, Folch wash procedure [28] by Lepage and Roy [24]. This method led to an increase in total fatty acids for plasma, faeces, bile and rat liver as compared with the Folch wash. They concluded that the direct esterification method was more efficient than the prevailing methods because of its simplicity, rapidity and higher accuracy.

Bitman [25] applied the direct transesterification method using acetyl chloride in methanol to human and cow milk fat and compared it with the NaOH–methanol, BF_3–methanol and acetyl chloride–methanol methods for the lipids extracted by the traditional Folch wash. The results compared well for most fatty acids in cow milk. The only exception

was a significantly lower mass percentage of C_4 acid by the direct transesterification as compared with the other methods. This derivatization technique was comparable to the BF_3–methanol method applied to Folch extracts for the analysis of human milk fatty acids. Browse *et al.* [29] used hot methanolic hydrochloric acid to digest the leaf tissue and to convert the fatty acids directly to FAMEs. The FAMEs were then extracted into the organic layer and analyzed by GC. This procedure resulted in a lower (10–20%) yield of fatty acids as compared with the conventional extraction, saponification and methylation steps to obtain FAMEs.

2.5. Preparation of esters other than methyl

Esters other than methyl can be prepared for a variety of reasons [4], *e.g.*, butyl esters [30,31] for the analysis of short-chain fatty acids present in dairy fats. The wide range of fatty acids (C_4–C_{26}) present in dairy fat and the volatility and solubility of the short-chain fatty acids require special attention in the preparation of FAMEs and GC analysis. The best methods for transesterification of such fats would be those in which no heating or solvent evaporation (which could result in the loss of volatile fatty acids) or extraction with water (resulting in the loss of soluble short-chain fatty acids) is involved. The Christopherson and Glass method [32] using sodium methoxide in methanol is generally recommended; however, the preparation of fatty acid butyl esters has been shown to give better recoveries of short-chain fatty acids. The method was discussed in detail by Iverson and Sheppard [30,31].

It is apparent that most of the reagents discussed above would serve the purpose depending on what type of sample is to be analyzed. However, care must be taken to follow the method exactly as specified because even a minor modification such as the use of cold instead of tepid water for extraction of the reagent [8], shaking manually instead of vortex mixing the solution [8] or heating for an extra few minutes [14] could account for an inaccurate fatty acid composition. With the newer reagents, especially where no extraction of the transesterification reagent is involved, their long-term effect on the GC column should be assessed. When introducing a new reagent it is not always practical to test its prolonged use on the GC column. To inject a sample 80 times into the GC system just to show the detrimental effect of excess of transesterification reagent on the GC column [3], even after predicting the effect, would be a misuse of resources. Hence it would be useful to receive information from different workers using such new reagents. The accuracy of the method should be demonstrated on primary standards, short- and long-chain and polyunsaturated fatty acids. It should be carefully assessed whether the method results in a preferential loss of certain fatty acids or causes the formation of conjugated or different geometric isomers. This point cannot be more stressed, taking into consideration that the conjugated linoleic acid (CLA), especially the 9-*cis*,11-*trans*-isomer, has been shown to exhibit anticarcinogenic activity [33–35]. CLA, which occurs in a number of foodstuffs (cheeses, milk fat, beef, etc.) has mostly been determined by the GC analysis of the FAMEs [33–36]. The differing amounts of CLA reported in the same foodstuffs could be due to a number of factors [34,36]; a possible reason could be that CLA was either formed or destroyed during esterification. We are currently assessing various esterification methods to study their applicability to correctly determine the different isomers of conjugated dienoic fatty acids present in dairy and meat products. The preferred reagent is that which does not cause any positional or geometric isomerization of the double bonds and gives a total recovery of the short-chain fatty acids.

3. GC COLUMNS AND STATIONARY PHASES

The superior resolution capability of capillary columns allows the routine identification of fatty acids on the basics of retention time alone, especially if the analysis is performed with stationary phases of different polarities. A wealth of data on the retention times and related parameters of most common fatty acids are available [37–39].

The use of packed columns for the analysis of fatty acids in food products and other samples is well known. Packed GC columns are still acceptable for preliminary analysis or screening procedures, but the increasing concern for the detection of specific fatty acid components in plant and animal tissues requires a much higher resolving power. Problems in maintaining a steady flow and the development of multiple flow paths of the carrier gas in packed columns limit the total column length to

about 6 m. On the other hand, the permeability of capillary columns allows for columns up to 100 m long, with *ca.* $4 \cdot 10^5$ theoretical plates [40].

The development of fused-silica capillary columns was a breakthrough in GC. Capillary columns made of stainless steel or borosilicate glass exhibited similar polarities and elution properties to modern fused-silica supports [41]. Stainless-steel or glass supports, however, presented problems of chemical reactivity or fragility, respectively [42]. Fused-silica columns provide a chemically inert support for the stationary phase. Modern fused-silica capillary columns are treated with a polyamide to decrease the possibility of damage due to an increase in flexibility. As this is essentially an external coating, the process does not modify the column polarity.

Despite their long history and analytical superiority, WCOT capillary columns have been widely accepted only in the last decade. Difficulties were based on the fact that they can be easily overloaded, resulting in a considerable decrease in resolution and quantification capabilities. Capillary columns demand more careful laboratory practices, a higher detector response (for sharp and rapidly eluting peaks) and a more sensitive detector for small sample sizes. Ironically, capillary columns have been criticized for giving "too much information" [42].

Resolution is a function of the square root of the length of the column. Therefore, large increases in column length (and retention times) will be required in order to improve the resolution significantly if other chromatographic conditions remain unchanged. Thus, most analyses are performed with an intermediate column length (*e.g.*, 30 m). The use of longer columns would only be necessary for very complex mixtures (about 100 individual components or more) and special applications (discussed later).

The inner diameter of the column also determined the column efficiency, retention time and column capacity. For columns with identical stationary phases, the smaller diameter would provide better peak resolution. In general, and when sample overloading is not a problem, the use of columns of 0.25 mm i.d. is recommended.

In theory, the only part of the column that will interact with the injected sample is the stationary phase. Therefore, the film thickness of the stationary phase is a primary factor in determining the retention of the sample components. Thick films will produce longer retention times and better resolution. Thick films are recommended for volatile solutes such as short-chain (*i.e.*, C_2–C_8) fatty acids. Most analyses, however, can be accomplished with the standard film thickness of 0.25 mm and an i.d. of 0.32 mm.

3.1. Polar versus non-polar stationary phases

While column selection in packed-column GC was crucial to obtaining satisfactory results, the greater resolution capacity of capillary columns made column selection less important. Most of the commercially available columns offer satisfactory separations of major fatty acids. In fact, capillary columns coated with a few stationary phases have replaced dozens of different types of packed columns [40]. Table 1 lists the frequently used stationary phases for capillary columns. Careful column selection in capillary GC is still important for special applications, as will be described later. As a general rule, the use of the least polar phase which provides satisfactory results is recommended. Non-polar phases exhibit superior lifetimes over polar columns.

Capillary columns are commercially available with a variety of stationary phases of different chemical compositions. A convenient way of indicating a GC column characteristic is the column polarity index (CP), which ranges between 0 and 100, from the extremely non-polar (squalene, CP = 0) to the most polar material available (cyanoethylsilicone, CP = 100) [43]. Methylsilicones are among the most widely used non-polar stationary phases, and they were the first to be developed for capillary GC [41]. Some of the advantages of methylsilicone columns are high thermal stability, a wide range of operating temperatures and chemical inertness. Most FAME mixtures can be separated with methylsilicone columns. In particular, very low polarity phases such as purified methylsilicone are preferred. On non-polar columns FAMEs are eluted in order of their boiling points, and the unsaturated components elute ahead of the corresponding saturated component of the same chain length. This elution order is the reverse of the elution order in columns with intermediate and highly polar stationary phases [41].

TABLE 1

CHEMICAL COMPOSITION OF MOST COMMONLY USED STATIONARY PHASES FOR CAPILLARY GC OF FOODS

Chemical composition	Brand names	Polarity[a]	Applications
100% Cyanopropylsilicone	CP-Sil-88 (Silar-10c)	VP	Soybean oil [61]
100% Cyanoethylsilicone oil	SP-2340, OV-275	VP	Milk fat [51], hydrogenated vegetable oils [62]
Methylsilicone polymer, 25% Cyanopropyl–25% phenyl–50% methyl	OV-225, DB-225, SP-2300	P	Meat fat [67]
Methylsilicone polymer, 1% vinyl–5% phenyl	SE-54	NP	Kernel oil [84]
Methylsilicone	SPB-1	NP	Fish oils [79]
	SPB-5	NP	Seed oils [58]
68% Biscyanopropyl–32% dimethylsiloxane	SP-2330	VP	Butter oil, vegetable oils [62]
	SP-2560	P	Hydrogenated vegetable oils [62]
Polyethylene glycol	DB-WAX, Supelcowax-10, Omegawax, Carbowax 20M	P	Yeasts [48], butterfat [66], marine oils [73], [68], blackcurrant seed oil [83], soybean oil [65] furan fatty acids [75], cottonseed oils [55]
95% Dimethyl–5% diphenylpolysiloxane	DB-5, SPB-5, CP-SIL 8CB	NP	Cottonseed oil [55]
100% Dimethylpolysiloxane	DB-1, Rt-1, SPB-1, SP-2100, OV-1, OV-101, CP-SIL 5CB	NP	Yeasts [48]
86% Dimethyl–14% cyanopropylphenylpolysiloxane	DB-1701	P	Yeasts [48]
Acidified PEG	Nukol, FFAP	P	Dairy products [51,53]

[a] VP = Very polar; P = polar; NP = non-polar.

The major drawback with non-polar phases is the co-elution of some unsaturated FAMEs. For instance, linoleic acid (18:2ω6)[a] partially overlaps with 18:1 and 18:3 fatty acids (Fig. 1). C_{20} and C_{22} fatty acids present similar overlapping problems [41]. Because many C_{18} and C_{20} fatty acids have important nutritional roles, polar or intermediate stationary phases are more often the choice in the analysis of food products.

Stationary phases of intermediate polarity, such as polyethylene glycol (PEG, Carbowax 20M) are widely used in the GC of FAMEs. Carbowax 20M is especially suitable for the separation of PUFA and it is being tested in an official method of the Association of Official Analytical Chemists for the analysis of lipids of marine origin [41]. In polar columns, each group of fatty acids, *e.g.*, 18:0, 18:1ω11, 18:1ω9, 18:1ω7, 18:2ω6, 18:3ω6, 18:3ω3 and 18:4ω3, will elute in this order (Fig. 2) [44]. This sequence would expand with increasing column polarity so that the more unsaturated FAMEs will elute after the next highest even-carbon saturated acid (in this example 18:4ω3 will elute after 20:0). In contrast to methylsilicone phases, PEG phases have lower temperature stability. A maximum of 250°C is the recommended temperature for isothermal GC on recently developed high-molecular mass PEG phases.

None of the polar or non-polar columns can completely separate all fatty acids normally present in a complex mixture. Therefore, a laboratory can benefit from the operation of two columns with different polarities.

[a] For the nomenclature used for fatty acids, consider the example 18:2ω6. In this notation, 18 refers to the total number of carbon atoms, 2 is the number of ethylenic bonds, and ω6 corresponds to the number of carbons from the center of the ethylenic bond furthest removed from the carboxyl group up to and including the terminal methyl group.

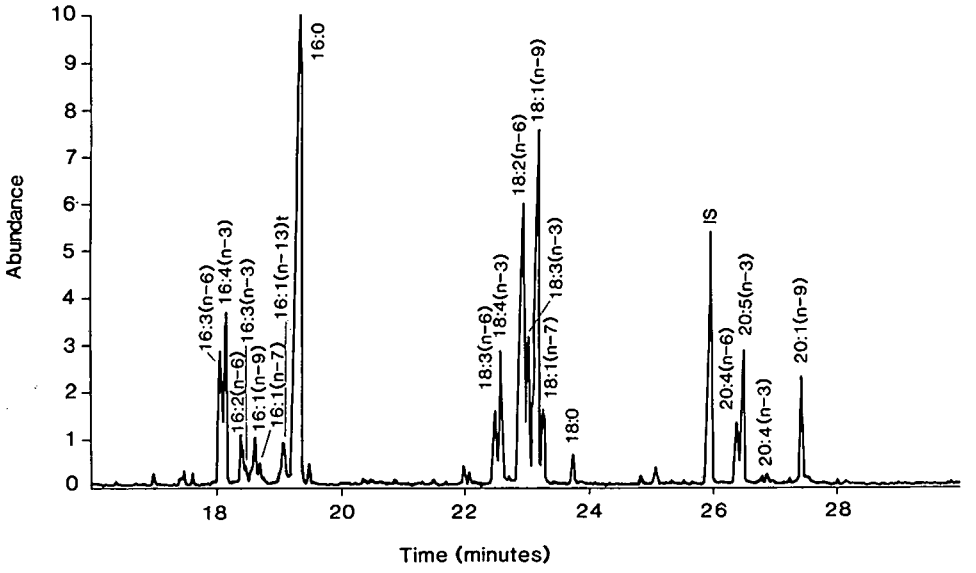

Fig. 1. Reconstructed chromatogram of FAMEs from the unicellular alga *Tetraselmis suecica*, obtained by GC–mass spectrometry using a 50-m methylsilicone fused-silica capillary column. From Volkman *et al.* [74].

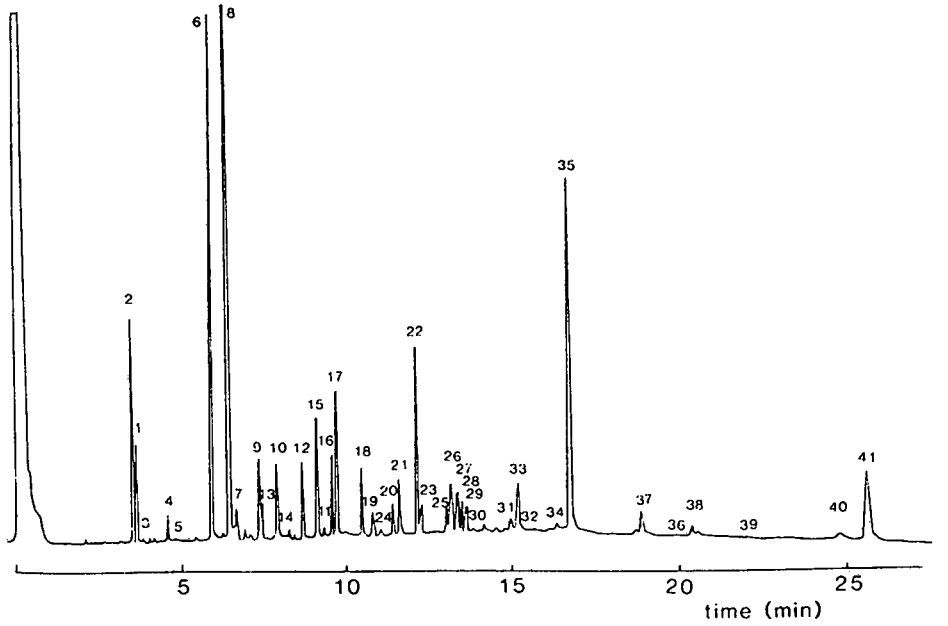

Fig. 2. Separation of FAMEs from the mussel *Mytilus galloprovincialis* on a GC capillary column coated with Silar 5CP. Peaks: 1 = 4,8,12-trimethyl-13:0; 2 = 14:0; 3 = 14:1ω5; 4 = iso-15:0; 5 = 15:1ω7; 6 = 16:1ω7; 7 = iso-16:0; 8 = 16:1ω5; 9 = 16:2ω4; 10 = 16:3ω4; 11 = 16:3; 12 = 16:4ω1; 13 = 17:0; 14 = 17:1ω8; 15 = 18:0; 16 = 18:1ω9; 17 = 18:1ω7; 18 = 18:2ω6; 19 = 18:2ω4; 20 = 18:3ω6; 21 = 18:3ω3; 22 = 18:4ω3; 23 = 18:4ω1; 24 = 19:1ω8; 25 = 20:1ω13; 26 = 20:1ω11; 27 = 20:1ω9; 28 = 5,11 20:2; 29 = 5,13 20:2; 30 = 20:2ω6; 31 = 20:3ω6; 32 = 20:3ω3; 33 = 20:4ω6; 34 = 20:4ω3; 35 = 20:5ω3; 36 = 21:5ω3; 37 = 7,15 22:2; 38 = 22:3ω6; 39 = 22:4ω6; 40 = 22:5ω3; 41 = 22:6ω3. After Christie *et al.* [44].

4. APPLICATIONS IN FOOD ANALYSIS

4.1. Microbiology

The GC of fatty acids is a useful technique in microbial chemotaxonomy [45–47]. In some instances significant and consistent differences in fatty acid compositions were used to identify genus, species and even strains of valuable microorganisms in the food industry [48]. Most bacteria contain a relatively simple fatty acid composition. Bacterial fatty acids normally are no longer than 18 carbons and have no more than one double bond [49]. Therefore, the use of packed columns for the analysis of fatty acids of microbial origin is still common. For example, a short GC column packed with SP-1200 and 1% H_3PO_3 was used in a rapid and simple procedure to determine concentrations of short-chain volatile fatty acids [50]. The use of a short column in this study significantly decreased the time of analysis of fermented products, and maintained an acceptable degree of resolution for underivatized short-chain (C_2–C_6) fatty acids. The screening or identification of different stains of fungi and bacteria requires a more refined separation and identification of fatty acids. Particular strains mainly differ in the proportion of their minor (< 1%) fatty acids [48]. In this comparative study thirteen strains of *Saccharomyces cerevisae* were successfully classified by comparing their fatty acid profiles in a series of capillary columns of different polarities such as PEG, dimethylpolysiloxane (DB-1) and dimethylcyanopropylphenylpolysiloxane (DB-1701).

4.2. Dairy products

Dairy products and especially butterfat are a challenge for the chromatographer as they present a very complex mixture of acyl lipids. The fatty acid chain length spans from C_2 to C_{26}, including straight and branched structures, with zero to six ethylenic bonds, and *cis–trans* geometric isomers. An additional complication is the presence of short-chain free carboxylic acid which may be lost during sample preparation. Free volatile fatty acids (FVFAs) in dairy products are present at low concentrations, but the level of these acids increases during ripening and they are important organoleptic components.

FVFAs were determined in the past on packed columns, using a variety of liquid phases (see ref. 51 for older references). More recently, FVFAs were determined by GC in underivatized form using a relatively short (*i.e.*, 15 m) capillary column coated with an acidified PEG (Nukol) stationary phase using crotonic acid as an internal standard [51].

Even though packed columns give reduced information [52], it is still suitable for the analysis of dairy products such as when analyzing a fraction of the total fatty acids, or to give an estimate of the total content of acyl lipids. However, for a more detailed study, the use of capillary columns is essential. De Jong and Badings [53] used a 25-m fused-silica capillary column coated with the polar phase FFAP-CB and a 15-m column coated with FFAP for the complete separation of FFA in cheese and milk (Fig. 3).

A problem during the analysis of underivatized FFAs is their adsorption on the first part of the capillary column. This problem is particularly serious with long-chain FFAs (16:0, 18:0 and 18:1), leading to selectively low responses. The adsorption of these fatty acids can be prevented by the use of capillary columns with thicker (1.0 μm) stationary phases [53].

4.3. Vegetable oils

Seed oils may contain a large number of minor monounsaturated fatty acids and positional isomers. Although many of these fatty acids can be separated by conventional capillary GC, their structural elucidation requires a more complicated treatment or derivatization. For instance, GC–mass spectrometry with capillary columns of medium polarity (*e.g.*, PEG) was used to separate and locate the position of the double bonds of picolinyl ester derivatives of fatty acids in borage (*Borago officinalis*) seed oil [54].

Seed oils are also a source of many uncommon fatty acids such as cyclopropenoid, epoxy or conjugated acids. Cyclopropenoid fatty acids such as malvalic (2-octyl-1-cyclopropene-1-heptaenoic acid) and sterculic (2-octyl-1-cyclopropene-1-octanoic acid) are common constituents of the plants of the order Malvales [55,56]. Interest due to possible health hazards [57] prompted the need for identifying and determining cyclic fatty acids in edible oils. PEGs or stationary phases of higher polarity, such as dimethyldiphenylpolysiloxane, were successfully used for the separation of dimethyloxazoline derivatives of cyclopropenoid fatty acids [55].

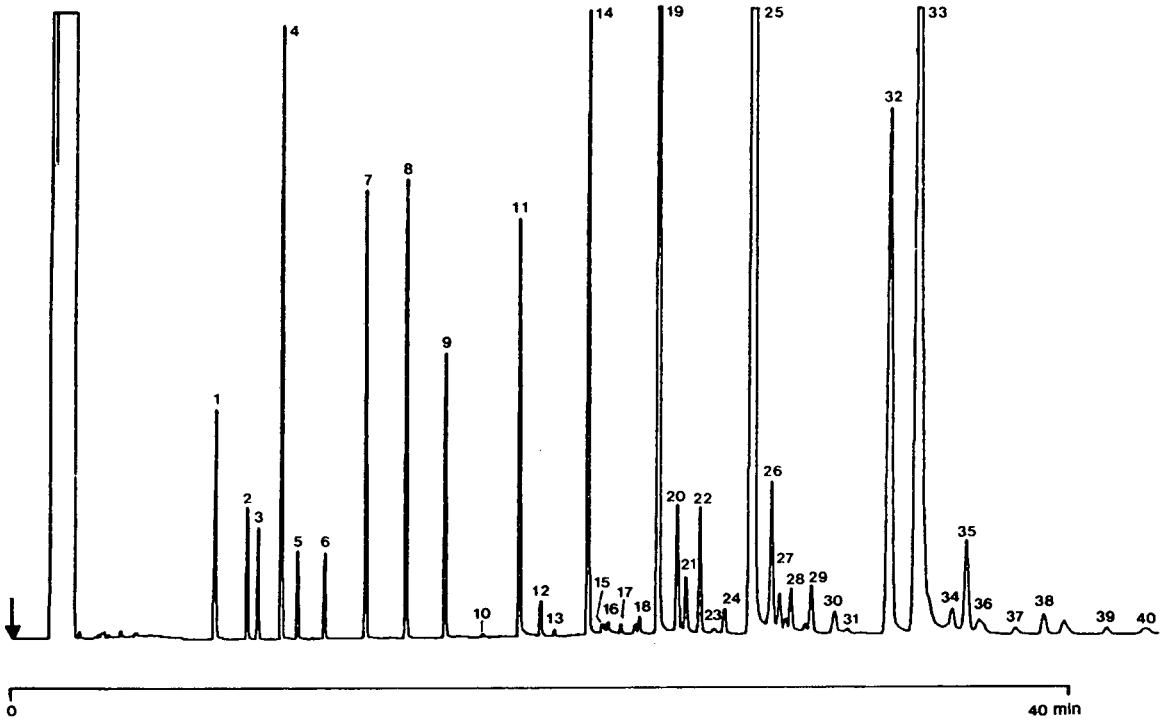

Fig. 3. Gas chromatogram of FFAs from cheese spiked with an FFA reference mixture (2:0, 3:0, 2-CH_3-3:0, 5:0, 3-CH_3-4:0 and 7:0). Peaks: 1 = C_2; 2 = C_3; 3 = 2-CH_3-C_3; 4 = C_4; 5 = 3-CH_3-C_4; 6 = C_5; 7 = C_6; 8 = C_7; 9 = C_8; 10 = C_9; 11 = C_{10}; 12 = C10:1; 13 = C_{11}; 14 = C12:0; 15 = C12:1; 16 = C_{13}-iso; 17 = C13:0; 18 = C_{14}-iso; 19 = C14:0; 20 = C14:1 + C15-iso; 21 = C_{15}-anteiso; 22 = C15:0; 23 = C15:1; 24 = C_{16}-iso; 25 = 16:0; 26 = C16:1; 27 = C_{17}-iso; 28 = C_{17}-anteiso; 29 = C17:0; 30 = C17:1; 31 = C_{18}-iso; 32 = C18:0; 33 = C18:1; 34 = C18:2; 35 = C18:2; 36 = C19:0; 37 = 18:3; 38 = C18:2 conjugated; 39 = C20:0; 40 = C20:1. After De Jong and Badings [53].

Epoxy fatty acids such as *cis*-12,13-epoxy-*cis*-9-octadecenoic acid, are present in high concentrations in a number of seed oils (*e.g.*, *Amaranthus cruentus*, a West African vegetable crop). These acids are susceptible to suffer a loss of functional groups and show poor recoveries when subjected to standard methylation procedures. A mild and rapid preparation of FAMEs with a methanolic solution of sodium methoxide was developed for the GC of epoxy fatty acids, and the FAMEs were separated with a 30-m SPB-5 column of low polarity [58].

Special applications of capillary GC to vegetable oils include the determination of individual oils in a blend. This objective can be achieved by a statistical analysis of GC data on fatty acids and other components. This approach was tested with a blend containing two, three or six oils (*i.e.*, corn, soybean, sunflower, palm, cottonseed, palm kernel, sesame, coconut and olive oils) [59]. The column used to analyze the FAMEs was a 2-m glass column packed with 10% diethyleneglycol succinate (DEGS) on Chromosorb W.

Trans fatty acids are minor components in natural foods, but relatively large concentrations of C_{18} *trans* fatty acids are formed by the industrial hydrogenation of vegetable oils in the processing of margarine [60]; 8-*trans*,12-*trans*-18:2 is a common product of the partial hydrogenation of vegetable oils. The successful analysis of this type of sample could represent a chromatographic problem, especially when the aim of the analysis is unequivocally to identify and determine individual components. Polar capillary columns coated with cyanopropylsiloxane are recommended for this purpose [61,62]. Complete fatty acid separations, however, can only be achieved by complicated procedures involving

more than one analytical technique [63]. Such a complex analysis is illustrated by the work of Ratnayake *et al.* (Fig. 4) [62]. This work shows the incomplete separation of several *trans*-octadecenoic acids even with the use of a very long (100-m) capillary column coated with a polar SP-2560 stationary phase.

Thermal oxidation of oils also produces a number of artifacts. Special attention has been paid to the production of fatty acid dimers and high polymers with different degrees of polarity. The separation of these components requires the combined use of gas, high-performance liquid and thin-layer chromatography. A complex mixture of synthetic dimers was analyzed by both packed and capillary column GC [64]. The separation of non-polar fatty acid dimers and isomers presented a difficult task. The mixture could at the best be partially resolved using 50-m long, low-polarity columns (*e.g.*, SPB-1, methylsilicone) [64].

The sensitivity of modern capillary GC–FID permits the routine determination of the fatty acid composition of a large number of small seed tissues. This information facilitates the genetic selection and further genetic manipulation of the lipid composition of seed oils. To simplify the analysis and to reduce the costs of column replacement, routine determinations are usually done on packed columns [65]. Simplified one-step extraction–derivatization and transesterification coupled with the rapid determination of FAMEs permitted inexpensive screening procedures for up to 2000 samples per month [65].

The high resolving power and reproducibility of

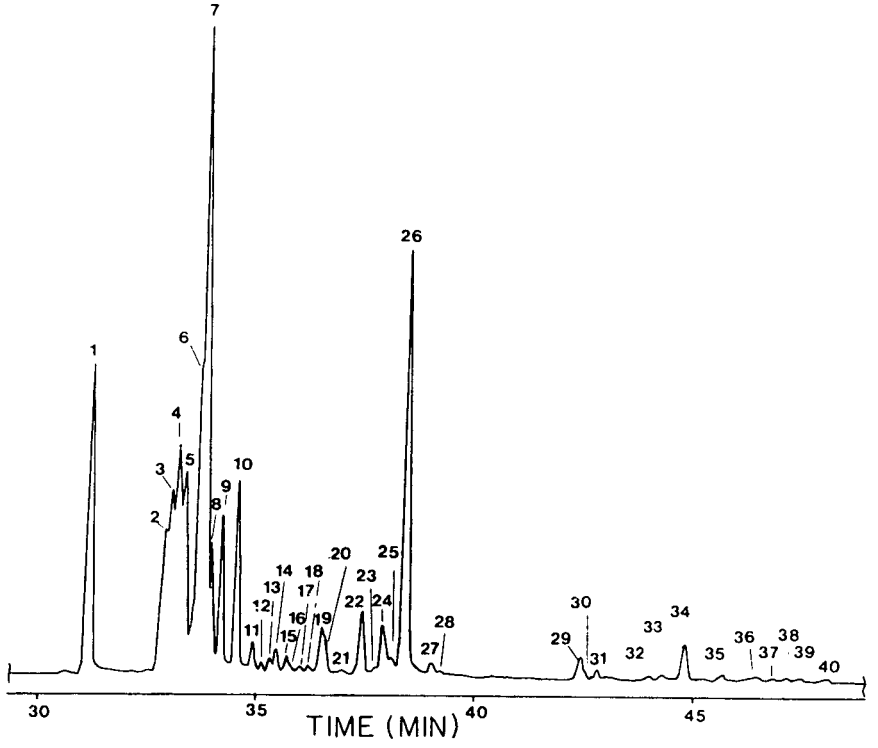

Fig. 4. Partial gas chromatogram of the C_{18} region of soybean oil margarine FAMEs on a 100-m SP-2560 capillary column. Peaks: 1 = 18:0; 2 = *trans*-6–8-18:1; 3 = *trans*-9-18:1; 4 = *trans*-10-18:1; 5 = *trans*-11-18:1; 6 = *trans*-12-18:1; 7 = 9-*cis*-18:1 + 13-*trans*-18:1 + *trans*-14-18:1; 8 = 10-*cis*-18:1; 9 = 11-*cis*-18:1; 10 = 12-*cis*-18:1 + 15-*trans*-18:1; 11 = 13-*cis*-18:1; 12 = *trans*–*trans*-18:2; 13 = 14-*cis*-18:1; 14 = *trans*–*trans*-18:2; 15 = 15-*cis*-18:1; 16 = *trans*–*trans*-18:2; 17 and 18 = *trans*/*cis*–*cis*/*trans*-18:2; 19 = *trans*-9-*trans*-12-18:2; 20 and 21 = *trans*/*cis*–*cis*/*trans*-non-methylene-interrupted (NMI)-18:2; 22 = *cis*-9-*trans*-12-18:2; 23 = ?; 24 = *trans*-9-*cis*-12-18:2; 25 = ?; 26 = *cis*-9-*cis*-12-18:2; 27 = 9-*cis*-15-*cis*-18:2; 28 = ?; 29 = 20:0; 30 = unidentified 18:3 isomer; 31 = *cis*-9-*cis*-12-*trans*-15-18:3; 32 = *trans*-9-*cis*-12-*cis*-15-18:3; 33 = *cis*-9-*trans*-12-*cis*-15-18:3; 34 = *cis*-9-*cis*-12-*cis*-15-18:3; 35 = 20:1; 36–40 = 18:2 conjugated ?. After Ratnayake *et al.* [62].

capillary columns helps in the design of methods for efficiently monitoring the quality of fats and oils. This approach is currently used to predict the adulteration of animal fats in butter–margarine blends with a high degree of certainty [66]. This type of analysis demands a positive identification of a wide variety of fatty acids ranging from short to long chain and *cis–trans* geometric isomers and also requires accurate determination. Modifications of this basic approach were recently developed in order to detect the presence of pork in processed meats [67] and to determine the content of clinically important fatty acids in fish oils [68].

4.4. Fish oils

Fish oils and in general marine lipids have a diverse fatty acid composition (typically an even-carbon chain length of C_{14}–C_{22} and with 0–6 methylene-interrupted double bonds). Marine lipids also contain minor amounts of less common fatty acids with non-methylene-interrupted double bonds, branched hydrocarbon chains or chain lengths longer than 22 carbons. Clinical and epidemiological studies in the last decade indicated that the consumption of fish or fish oils can improve or prevent certain human cardiovascular conditions [69]. This potential therapeutic property of marine lipids renewed interest in investigating the lipid contents and fatty acid compositions of fish and shellfish species around the world [70]. Studies were especially concentrated on the accurate determination of the content and availability of marine fatty acids of medical interest (eicosapentaenoic acid, EPA, 20:5ω3; and docosahexaenoic acid, DHA, 22:6ω3). The fatty acid compositions of many seafood products and edible algae and encapsulated fish oil products have been reviewed [71–73].

Typically, fatty acids of marine origin are analyzed with capillary columns of medium polarity, such as Carbowax 20M. Earlier work with packed columns presented serious problems of overlapping between different even-carbon fatty acids [39]. Of special interest is the coincidence of 22:1 with 20:5ω3 on medium-polarity packed columns, and 22:1 with 20:4ω6 on higher polarity packed columns [39]. There are a number of reports on the capillary GC of marine lipids using non-polar stationary phases [74]. Low-polarity capillary columns, however, present some partial overlapping problems, especially with the pairs 18:2ω6–18:3ω3 and 20:4ω6–20:5ω3. PEG columns, on the other hand, have shown reasonably good thermal and chemical stability [41]. They offer excellent resolution of all major fatty acids of marine lipids and of a number of minor but ubiquitous components of fish oils. For example, capillary columns coated with Carbowax 20M separated furan fatty acids of salmon roe [75], EPA geometric isomer artifacts in heated fish oils [76] and branched-chain fatty acids in fish oil concentrates [77].

5. QUANTIFICATION

Quantification errors can be introduced in many different ways and each step in the analysis of fatty acids has the potential for sample loss. Sources of problems for the determination of fatty acids by GC–FID arise during sample preparation (extraction and derivatization), on the injector, on the column, in the detector and during data collection.

Quantification errors originating as a consequence of column reactivity can be reduced by selecting highly pure stationary phases and inert supports. In all instances it is recommended to perform routine checks on selective adsorption or losses of FAMEs in the column. Calibration of the GC system should be performed by using FAME mixtures similar in composition to the sample being analyzed. Partial overlapping and co-elution of different components may generate other quantification problems [78].

Quantification of PUFA presents the additional problems of losses due to oxidation during sample handling. Losses of PUFA can be easily determined by comparing the fatty acid composition of the sample before and after total hydrogenation [39].

With capillary columns problems arise as a consequence of defective injection procedures. Christie [79] developed a series of simple and effective injection techniques, such as hot needle and cold trapping injection. He indicated that an injection temperature of about 375°C improved the reproducibility of the analysis without causing any adverse effect on the recovery of PUFA. Regardless of the preferred injection technique, the use of automatic injection is highly desirable for minimizing the introduction of random errors.

When the determination of FAMEs with FID re-

quires high accuracy, correction factors should be applied to compensate for the fact that the carboxyl carbon is not ionized during combustion [80]. In addition, the degree of unsaturation of a FAME mixture also requires the use of small correction factors. The magnitudes of these factors are greater for very short-chain or highly unsaturated fatty acids [79].

There are a few reports on quantitative aspects of the capillary GC of fatty acids. Not unexpectedly, the results of inter-laboratory studies have shown very poor reproducibility for the analysis of fatty acid mixtures containing a wide variety of molecules [80]. Inter-laboratory studies rely on the availability of reference samples and standards. Standardized FAME mixtures are available from the National Institute of Health and several commercial suppliers, but unfortunately they do not cover all areas of application.

A linear FID response is generally assumed for long-chain fatty acids. Therefore, absolute concentrations are determined by adding odd-chain saturated fatty acids as internal standards (*e.g.*, 17:0, 19:0 and 23:0). Recently, 24:1ω9 (nervonic acid) has been used as internal standard for the quantitative analysis of fish oils [81]. The addition of a single internal standard assumes no discrimination of the different fatty acids during the processes of isolation, derivatization and actual chromatography.

A more refined approach was recently developed, consisting in the use of a series of odd-chain fatty acid standards. The determination of a naturally occurring even-carbon fatty acid was compared with the two adjacent odd-carbon fatty acids ("bracketing" method) [82]. It has been demonstrated that this bracketing method is superior to the use of a single fatty acid as an internal standard [82].

6. CONCLUSIONS

GC is the method of choice for the determination of fatty acids. Food products contain a wide variety of lipids and their fatty acids differ in chain length and degree of unsaturation and in the presence of special functional groups and positional and geometric isomers.

Procedures for the derivatization of fatty acids need to be optimized to achieve good quantitative results. In general, most transesterification methods, whether acid- or base-catalyzed, or direct methods would meet the purpose depending on the nature of the sample to be esterified. New reagents and procedures, especially where no removal of the reagent is recommended, should be assessed for their long-term effect on the GC column.

Contemporary capillary GC offers excellent separations of most naturally occurring fatty acids. These capillary columns, especially those of medium polarity (*e.g.*, Carbowax 20M), and the nonpolar methylsilicone, successfully separate FAMEs ranging from C_2 to C_{24} and with 0–6 ethylenic bonds. Special applications, such as the separation of complex *cis–trans* fatty acid mixtures (characteristic of partially hydrogenated oils), cyclopropene or epoxy fatty acids (common in some seed lipids), require particular chromatographic conditions. These include the use of very long capillary columns (*e.g.*, 100 m) and/or more polar stationary phases composed of cyanoethyl- or cyanopropylsiloxanes.

7. ACKNOWLEDGEMENTS

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Review

Electrophoresis and chromatography of wheat proteins: available methods, and procedures for statistical evaluation of the data

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ABSTRACT

Analysis of gluten proteins from the wheat grain endosperm has long challenged the analytical chemist. Several hundred unique polypeptides are present, many in large polymers. This complexity, plus useful relationships of composition to genotype and quality, encouraged development and application of electrophoresis and chromatography for gluten analysis. We review the methods of polyacrylamide gel electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing and high-performance liquid chromatography available for study of wheat proteins. Singly and in combination, they provide rapid, reproducible, high-resolution separations based on size, charge, or surface hydrophobicity. As challenging and important as the analyses themselves, however, is interpretation of data. Subjective evaluation is sometimes possible, but statistical methods such as similarity scores, clustering, principal components, multiple linear regression, and partial least squares now are increasingly used for data analysis. We review the use of these procedures, and precautions necessary to avoid misinterpretation of data. Optimal evaluation of protein analytical data will enhance the value of such analyses in wheat breeding, marketing, and processing.

CONTENTS

The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned.

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1. INTRODUCTION

Wheat (*Triticum aestivum* L.) has long been a primary food source because of its productivity, adaptation to diverse environments, and good nutritional value. Wheat's popularity is also due to the excellent baked goods made from it. We can produce such products because of wheat's storage proteins. To the plant, these proteins are the nitrogen reserve for the germinating embryo. For us, however, they are an important part of the diet. These proteins, known collectively as gluten, also have unique properties. After wheat is milled and the resulting flour is hydrated, dough develops as proteins interact with each other and other flour constituents to form a continuous cohesive matrix. This gluten network is elastic, and retains carbon dioxide generated during dough fermentation. Upon baking, its expanded structure is set, resulting in porous, fine-textured products.

Because of the importance of gluten, and since variation in structure is associated with quality, understanding gluten's structure has long been important. In 1745, Beccari [1] described milling of wheat and gluten–starch separation, and attempts to characterize gluten. He concluded that such studies, "... relevant both to sickness and to health ...." should encourage others to investigate food. In a seminal report Osborne [2] described gluten's unique properties, and noted that only wheat has a protein so easily isolated. He separated gluten into two major protein classes, gliadin, soluble in aqueous alcohol solutions, and glutenin, insoluble in alcohol but soluble in dilute acid or alkali.

Osborne [2] and those who followed him have had a difficult task. Isolation and characterization of gluten's many components is one of the most difficult challenges faced by protein chemists. This is partly because gluten is so heterogeneous: since gluten consists of storage proteins, there are few constraints on its expression. Also, genes which code gluten were duplicated during evolution. Finally, bread wheat is hexaploid, having three closely related genomes. Thus, many polypeptides form gluten.

Gluten characterization is also difficult because of its atypical characteristics. Gluten is rich in glutamine, leading to hydrogen bonding. Hydrophobic amino acids are also abundant, contributing to insolubility of these proteins in water and buffers. Most gluten proteins are either prolamins (gliadin in wheat), soluble in aqueous solutions of alcohols, or glutelins (glutenin in wheat), insoluble in alcohol but soluble in acid, alkali, denaturing agents, detergents, or reducing agents. At least three classes of monomeric gliadins exist (α/β, γ and ω), which vary in size and composition. In contrast, glutenin is a polymer of two major [low-molecular-mass (LMW)

high-molecular-mass (HMW)] plus minor subunit classes, joined through disulfide bonds into proteins with relative molecular masses (M_r) ranging into the millions.

It is now possible to better isolate and characterize gluten, and to relate its composition to quality and genotype. This is possible because of improved electrophoretic and chromatographic methods. Each new method showed gluten to be more complex than had been indicated by earlier techniques, and indicated the need for still better methods. As data became more complex and easier to acquire, it also became apparent that visual data evaluation is not sufficient. Computer-assisted statistical procedures are needed to fully reveal information in the results.

We will here review advances in electrophoresis and chromatography for gluten fractionation, and show how these are being used. Other reviews [3–11] summarize earlier progress, and our knowledge of wheat protein composition, structure and functionality; a recent Proceedings volume also provides a useful overview of these topics [12]. We will also review an important related topic: methods to evaluate electrophoresis and chromatography data. Visual evaluation is sometimes adequate, but much information is not readily apparent. Computer-assisted statistical methods of data interpretation promise to enhance the value of chromatography and electrophoresis for analysis of wheat and its proteins.

2. ELECTROPHORETIC METHODS

Electrophoresis was the first procedure to reveal the composition of gluten. Electrophoretic mobility depends on net charge, resulting from ionizable amino acids, and on polypeptide size. Gluten proteins have few basic or acidic residues, giving them low charge/mass ratios, but electrophoresis can separate them in several modes: (a) both size and charge influence mobility; (b) charge differences can be suppressed by a detergent such as sodium dodecyl sulfate (SDS) so separations depend only on size, as in SDS polyacrylamide gel electrophoresis (PAGE); and (c) size does not influence mobility, as in isoelectric focusing (IEF), since proteins migrate in a pH gradient to positions where they are electrically neutral. These applications will be reviewed in this section.

2.1. Moving-boundary electrophoresis

Early attempts to analyze gluten by electrophoresis were by moving-boundary electrophoresis in an open tube. With gluten, this method was originally limited by gluten's solubility. Jones *et al.* [13] described buffers permitting separations of gluten. One buffer, pH 3.1 aluminum lactate (and variations involving other lactate salts), became a highly successful solvent for gliadin electrophoresis. Using it, gluten was shown to contain at least five gliadins and one glutenin [13]. Gluten composition varied qualitatively and quantitatively among and within wheat species. This method thus gave the first real evidence that gliadin and glutenin were themselves heterogeneous.

2.2. Starch gel electrophoresis

Lactate buffer was later combined with zone electrophoresis in starch gels [14–17], which stabilize electrophoretic separations. Starch gel electrophoresis (SGE) remains valuable for fractionating gliadins, showing them to be more heterogeneous than originally known. Today, 20–30 bands may resolve by SGE. Woychik *et al.* [17] first proposed that gliadins be subclassified as ω, γ, β and α, based on increasing mobility. SGE also readily distinguishes gliadins from albumins, which have greater mobilities, and from glutenin, which is polymeric and too large to enter the gel or give distinct bands.

SGE of wheats revealed major differences in gliadin compositions [18,19], permitting varietal identification. Standard methods have been proposed [20], and varietal identification by gliadin SGE is still used today [21,22]. Albumins and globulins also separate well by SGE, differentiating genotypes and classes [18]. SGE uses simple equipment and a non-toxic support. Nevertheless, resolution is variable, and it is difficult to reproducibly prepare starch gels, which are not very stable.

2.3. Polyacrylamide gel electrophoresis

Polyacrylamide has generally replaced starch as the medium for gel electrophoresis of wheat proteins. Separations are analogous, but resolution of PAGE is generally better. Polyacrylamide gels can be prepared reproducibly and are stable, and thin

slabs can be prepared and used, enhancing resolution because of better heat dissipation. Gel pore size and concentration can also be varied to separate proteins of different sizes.

Wheat proteins were first separated by PAGE in 1963 [23]. Since then, techniques have been modified to improve resolution, especially of gliadins [21,24–30]. An example (Fig. 1) compares Canadian spring wheats on a 6% gel (200 × 150 × 3 mm) [31]. Cultivars are easily differentiated by characteristic fingerprints. PAGE of albumins and globulins also can discriminate among genotypes [21,32,33], but differences are more quantitative than qualitative.

Most PAGE separations of gliadins use pH 3.1

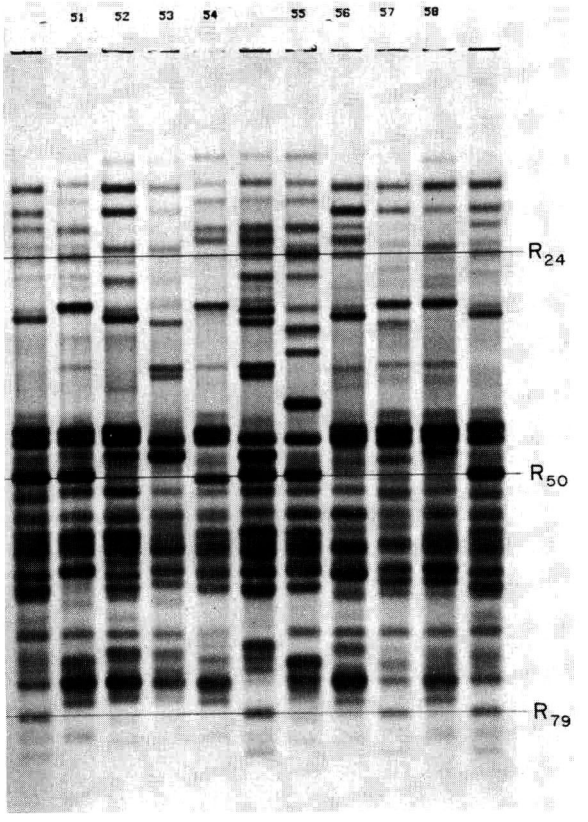

Fig. 1. Polyacrylamide gel electropherograms (6% gel, 200 × 150 × 3 mm) in pH 3.1 aluminum lactate–lactic acid buffer of gliadins extracted with 70% ethanol from several Canadian common spring wheat cultivars. Horizontal lines denote migration distances of reference bands of the standard cultivars Marquis (outside slots) and Neepawa (center), used to standardize gels and increase precision of the mobility data. From Sapirstein and Bushuk [31].

aluminum lactate-lactic acid buffer and a uniform gel. Good resolution is also achieved with acetic acid–glycine buffer [34] or at alkaline pH [32]. Gradient PAGE is also very successful: polyacrylamide concentration increases with migration distance, reducing mobilities and sharpening bands [21,25]. Precast gels can give especially convenient, reproducible, and rapid results [25].

Resolution, precision, and speed of wheat protein PAGE have also improved. Lookhart *et al.* [35] showed that sodium lactate buffer can be used for gliadin PAGE, and that resolution is generally best at 7–10°C. Clements [36] also showed that lactate buffer without aluminum gives good resolution of gliadins extracted with ethylene glycol. Variables such as gel thickness; buffer type, pH, and ionic strength; temperature; catalyst and apparatus design can also be adjusted to optimize gliadin PAGE for varietal identification [37].

Rapid PAGE methods are especially useful in determining wheat varietal purity and for selection and marketing, where many analyses must be done. One such method can identify varieties within 1 h [38,39]. Gliadins are extracted with 6% urea or ethylene glycol, and separated for 9 min in 75 × 35 × 1 mm gradient gels in pH 3.1 sodium lactate buffer. Discrimination between varieties is as good as on standard-sized gels. Labor-saving techniques of protein extraction and application are also useful for screening samples [40], and standardized PAGE methods for cultivar identification [41,42] can reduce variation between laboratories.

Sapirstein and Bushuk [31,43,44] significantly improved the precision of PAGE for varietal identification. Gels (as in Fig. 1) are photographed, and migration distances of bands are determined and entered into a computer with a digitizing tablet. Positions of bands are then normalized based on mobilities of three reference bands in standard cultivars. Band intensities are also estimated and entered into the data base. Unknown samples are then compared to stored data for known varieties. This process can be automated, and gives precise, accurate PAGE varietal identification.

2.4. Isoelectric focusing

Whereas gel electrophoresis was borrowed from clinical disciplines, IEF was first used to fractionate

wheat albumins and globulins [45,46]. Proteins separate in a pH gradient according to differences in isoelectric points, complementing other electrophoresis procedures. High resolution results, sometimes superior to that of PAGE [47,48]. For gliadins, Wrigley [45,46] observed several bands with isoelectric points of 5–8, and confirmed that IEF patterns differ among varieties [21,25]. The high cost of ampholytes may limit use of IEF for routine analyses, but this is less serious with miniature gels. IEF is also common as the first dimension in two-dimensional (2D) procedures (see below).

An important recent IEF variation uses Immobilines (*i.e.*, immobilized ampholines) to stabilize the pH gradient. This technique appears to have been used only once for wheat proteins, which focused without cathodic drift in a pH 4–10 gradient as the first separation in a 2D separation [49]. This eliminated the need to use two IEF techniques, including non-equilibrium pH gradient electrophoresis (NEPHGE) to resolve basic proteins.

The other major advance in IEF of wheat proteins is free-flow preparative IEF [50,51]. This procedure uses a Rotofor apparatus (Bio-Rad Labs., Richmond, CA, USA) consisting of a cylindrical chamber with 20 compartments divided by membranes that maintain separations. After IEF using pH 3–10 ampholytes, fractions are recovered from each chamber.

Results of such a separation of several hundred mg extracted gliadin and glutenin are shown in Fig. 2 [51]. SDS-PAGE revealed an excellent separation, with only slight overlap between fractions. Results also clearly show native glutenins of different compositions.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

While SGE, PAGE and IEF provided excellent separations of gliadins, albumins, and globulins, they generally did not separate glutenin (or its subunits) well because of its polymeric nature, high M_r, associative tendencies, and poor solubility. The advent of SDS-PAGE [52] made characterization of glutenin possible. Proteins are turned to random coils, and charge differences are eliminated by bound SDS. Separations occur almost totally on the basis of size, and M_r can be estimated by comparison to mobilities of standard proteins.

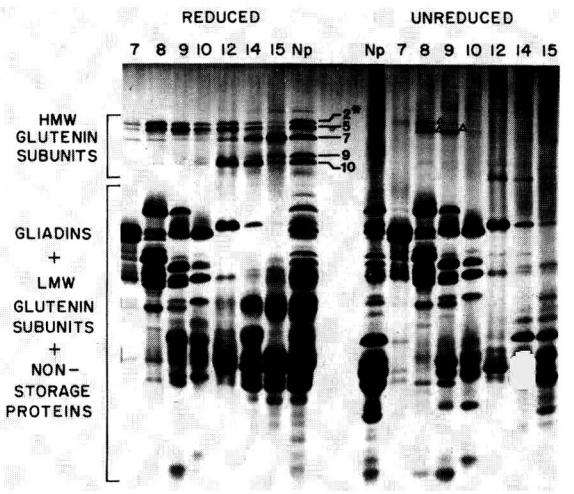

Fig. 2. SDS-PAGE patterns of reduced (left) and unreduced (right) proteins extracted (using a pH 8 Tris–HCl buffer containing urea and SDS) from the wheat variety Neepawa and fractionated by free-flow preparative IEF. Numbers at the top of the gel are fraction numbers; Np is a total protein extract of Neepawa flour. From Ng *et al.* [51].

Bietz and Wall [53] first used SDS-PAGE to characterize gluten. Analysis without disulfide bond cleavage gave a streak from the origin, but adding a reducing agent revealed discrete zones from M_r 10 000 to *ca.* 140 000. Other studies, however, show that M_r estimates vary considerably with procedure. M_r estimates of some HMW glutenin subunits may be nearly twice actual M_r. This anomaly has not been well explained, and is not well understood.

Using SDS-PAGE, Bietz and Wall [53] resolved gliadin into components of apparent M_r 30 000–80 000 (Fig. 3). The largest M_r 60 000–80 000 were ω-gliadins, and α-, β- and γ-gliadins formed overlapping zones (M_r 30 000–40 000) below ω-gliadins. Early studies also showed albumins and globulins to be very heterogeneous, most with $M_r < 40\,000$. Better separations of albumins and globulins were later achieved [54].

SDS-PAGE results for glutenin (Fig. 3) [53] were even more revealing. It contained both HMW (M_r 100 000–140 000) and LMW (M_r 30 000–50 000) subunits joined through disulfide bonds. The large size of HMW subunits and their occurrence only in glutenin, wheat's strength protein, indicated a role in breadmaking. This was confirmed by SDS-PAGE of wheat aneuploids [55], which showed

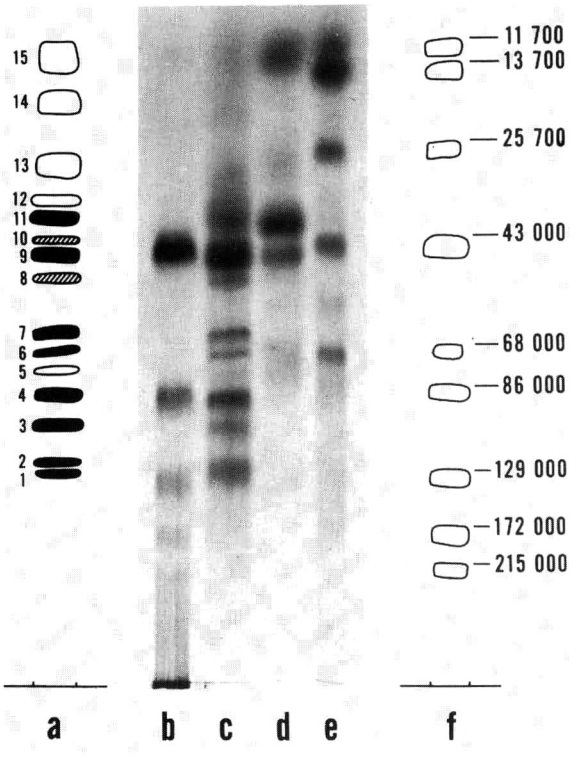

Fig. 3. SDS-PAGE analyses of wheat gliadin (d), glutenin (a and c), and standard protein mixtures (b, e, f). Apparent M_r are indicated to the right of the gel. From Bietz and Wall [53].

them coded by genes on chromosomes (1B and 1D) associated with breadmaking quality.

Initial SDS-PAGE studies showed minimal variation among bread wheat varieties [55], but higher-resolution methods later showed considerably greater differences [56–58]. SDS-PAGE may thus be useful for cultivar identification [21,59,60]. SDS complements methods based on charge, and shows apparent M_r rapidly and precisely if suitable references are used [61].

Use of higher resolution SDS-PAGE revealed major differences in HMW glutenin subunit compositions. Four or five HMW subunits occur in a variety, and 20 or more different HMW subunits are in all wheats. These subunits indicate alleles related to breadmaking quality [62–64]. They can also be analyzed in total protein extracts [58,65], simplifying sample preparation and making SDS-PAGE useful for selection during breeding.

Glutenin also has LMW subunits with apparent M_r similar to gliadins (Fig. 3). This M_r similarity, and the difficulty of separating gliadin from glutenin, originally made SDS-PAGE characterization of LMW glutenin subunits difficult. Newer procedures, however, give excellent resolution of LMW glutenin subunits. In one procedure [66,67], unreduced total protein seed extracts are first electrophoresed so gliadins migrate from the origin. A strip of gel next to the origin, containing unreduced glutenin, is then removed, equilibrated with buffer containing reducing agent, loaded onto a new gel slab, and again subjected to SDS-PAGE. From 7 to 16 LMW glutenin subunits resolve, free from overlapping gliadins. Varietal differences among these subunits may relate to pedigree and quality. An alternative two-step one-dimensional method [68] analyzes gliadins as well as HMW and LMW glutenin subunits. Unreduced proteins are first separated by acid PAGE, giving good separations of gliadins. A gel strip from below the sample wells is then equilibrated with SDS and reducing agent, and electrophoresed by SDS-PAGE, separating HMW and LMW glutenin subunits free of overlapping gliadins.

Fig. 4 shows an example of results achievable today by SDS-PAGE in comparing glutenin subunit compositions from various wheats [69]. Glutenin was purified by a rapid dimethyl sulfoxide extraction procedure [70], reduced, pyridylethylated to stabilize cysteines, and analyzed by gradient SDS-PAGE with silver staining [71]. Subunits of 37 M_r classes, from 30 000 to 116 000, were revealed. Such resolution is due both to the improved electrophoresis procedure (a 0.75 mm thick 11.0–16.5% polyacrylamide gradient gel) and to the small amount of sample applied when silver stain is used. Excellent SDS-PAGE results on similar gradient gels were also reported by Marchylo and co-workers [72,73], who resolved 7–11 HMW and 25–32 LMW polypeptides from gliadin plus glutenin. This procedure could differentiate most varieties.

Rapid SDS-PAGE represents another major advance. When reduced wheat proteins are separated on 70 × 80 × 1 mm 12% polyacrylamide gels, separation time decreases from about 20 h (for 160 × 140 × 1.5 mm gels) to 2.5 h, and resolution is nearly as good [74]. Another rapid procedure uses even smaller (50 × 43 × 0.45 mm) gels with the Pharmacia PhastSystem [73]. Typical results are shown in

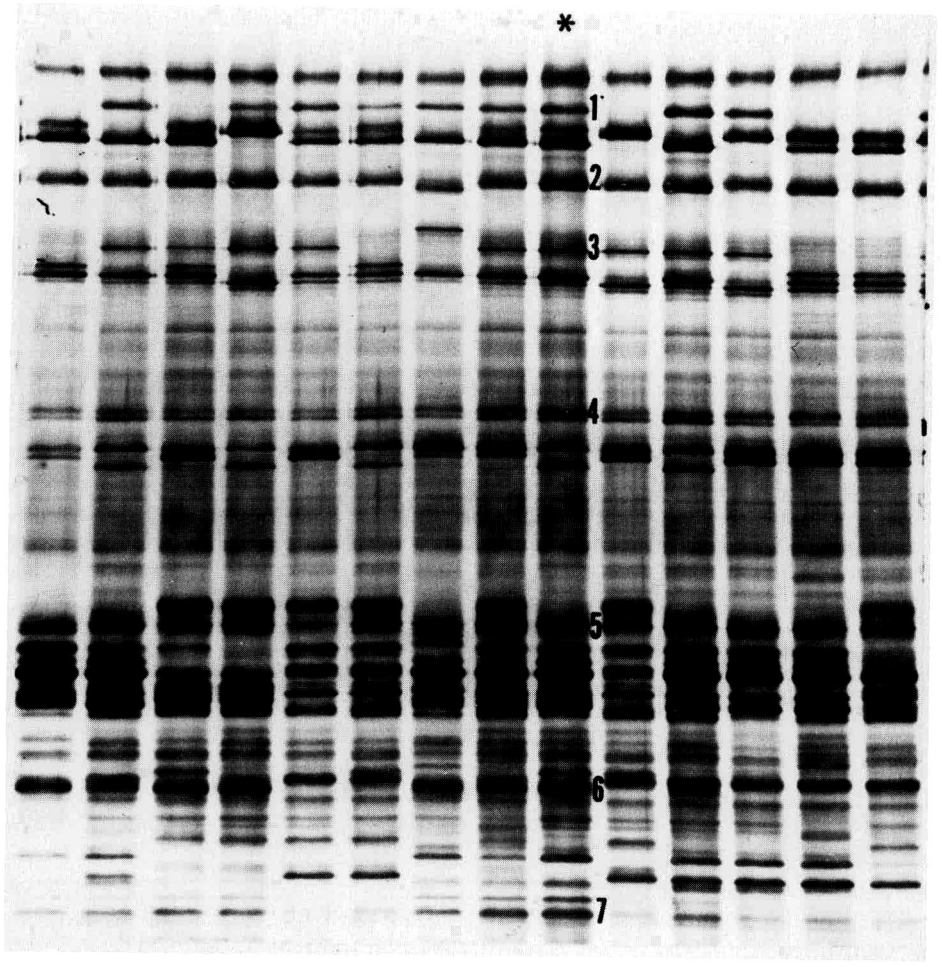

Fig. 4. SDS-PAGE separations of glutenin subunits from experimental wheat lines. Apparent M_r of numbered bands are: 1 = 115 300; 2 = 99 600; 3 = 87 900; 4 = 61 300; 5 = 45 300; 6 = 37 300; 7 = 32 000. From Graybosch *et al.* [69].

Fig. 5. Resolution by this method, with a 45 min separation and 44 min silver staining, is nearly as good as that on conventional large gradients gels. PhastGels distinguish most cultivars, and glutenin HMW subunits are readily identified. This procedure is effective for rapid analysis when samples are few or in non-standard situations.

SDS-PAGE can also provide valuable quantitative data. Kolster and Van Gelder [75] describe procedures for extraction, SDS-PAGE, staining, and densitometric quantitation of wheat proteins. HMW glutenin subunits can be both identified and quantified in one step.

Another interesting SDS-PAGE advance is electroendosmotic preparative electrophoresis [76]. Buffer flow between electrodes (electroendosmosis) moves electrophoresed proteins along a cylindrical gel, from which they are eluted and collected. Curioni *et al.* [76] used this method to isolate mg quantities of five pure HMW glutenin subunits in one step.

2.6. Two-dimensional electrophoresis

Any two electrophoresis procedures, especially when complementary separation modes are involved, can be combined to enhance protein resolu-

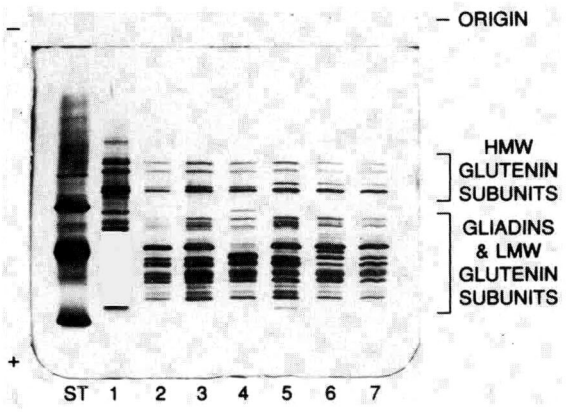

Fig. 5. Fast horizontal SDS-PAGE of wheat storage proteins using an 8–25% gradient PhastGel with Pharmacia's PhastSystem. Samples shown are: ST = standard mixture of known proteins; 1–7 = storage proteins from seven Canada Western Red Spring wheat cultivars. Proteins were extracted with 50% 1-propanol containing 1% dithiothreitol and 41 m*M* Tris–HCl, pH 8.0, and alkylated with 4-vinylpyridine before SDS-PAGE. From Marchylo *et al.* [73].

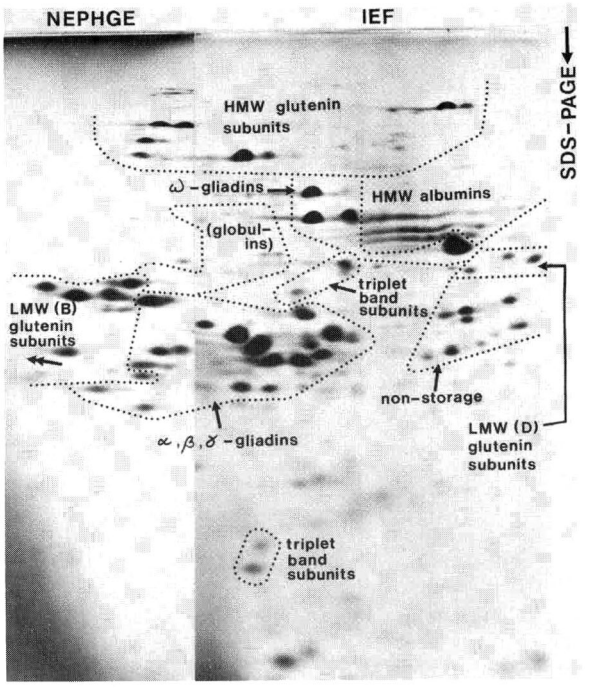

Fig. 6. Fractionation of wheat endosperm proteins by NEPHGE × SDS-PAGE and IEF × SDS-PAGE. The gels are overlapped to show a continuous pH gradient. The map is divided into areas according to biochemical and genetic properties of the proteins. From Payne *et al.* [84].

tion. The best separations of wheat proteins to date have been by 2D electrophoresis.

Wrigley and Shepherd [77,78] first separated wheat albumins and gliadins by 2D electrophoresis by combining IEF with SGE. Since these methods are complementary, single bands in one separation may further resolve in the other. Combined IEF and SGE also showed chromosomal locations of genes coding water- and chloroform–methanol-soluble wheat proteins [79]. Other 2D separations of albumins/globulins combine IEF with PAGE [47,80].

Other combinations of procedures also give excellent separations. For example, PAGE first in aluminum lactate buffer, pH 3.1–3.2, and then in Tris–glycine buffer at pH 9.2 separates albumins, globulins and gliadins [26,81,82], and helped locate their coding genes through aneuploid analysis. Both separations occur in one gel slab.

IEF combined with SDS-PAGE [83], however, generally gives highest resolution 2D separations. For example, Payne *et al.* [84] separated α-, β- and γ-gliadins, LMW and HMW glutenin subunits, and albumins and globulins by combining two IEF procedures, including NEPHGE to separate basic polypeptides, with SDS-PAGE (Fig. 6). Polypeptides of each class have similar size and charge, and group together on the gel. Using similar procedures, Lei and Reeck [85] resolved nearly 500 wheat proteins; Anderson *et al.* [86] achieved similar resolution. Such methods have clarified protein inheritance by locating coding genes [84,87].

Such complex data are, however, difficult to interpret: *e.g.*, rows of spots sometimes result, possibly being charge variants of a protein [88] arising through mutation. Proving such relationships is difficult, but proteins can be electroblotted from gels and sequenced [89,90].

The limit of resolution of 2D procedures has not yet been reached. Tkachuk and Mellish [91], using IEF plus SDS-PAGE, detected about thirteen hundred wheat albumins, globulins, gliadins, and glutenins. A recent 2D prolamin separation is shown in Fig. 7. Optimal staining (generally with silver stains) [92] also enhances resolution: smaller samples can be used, reducing interactions of proteins and making spots more compact.

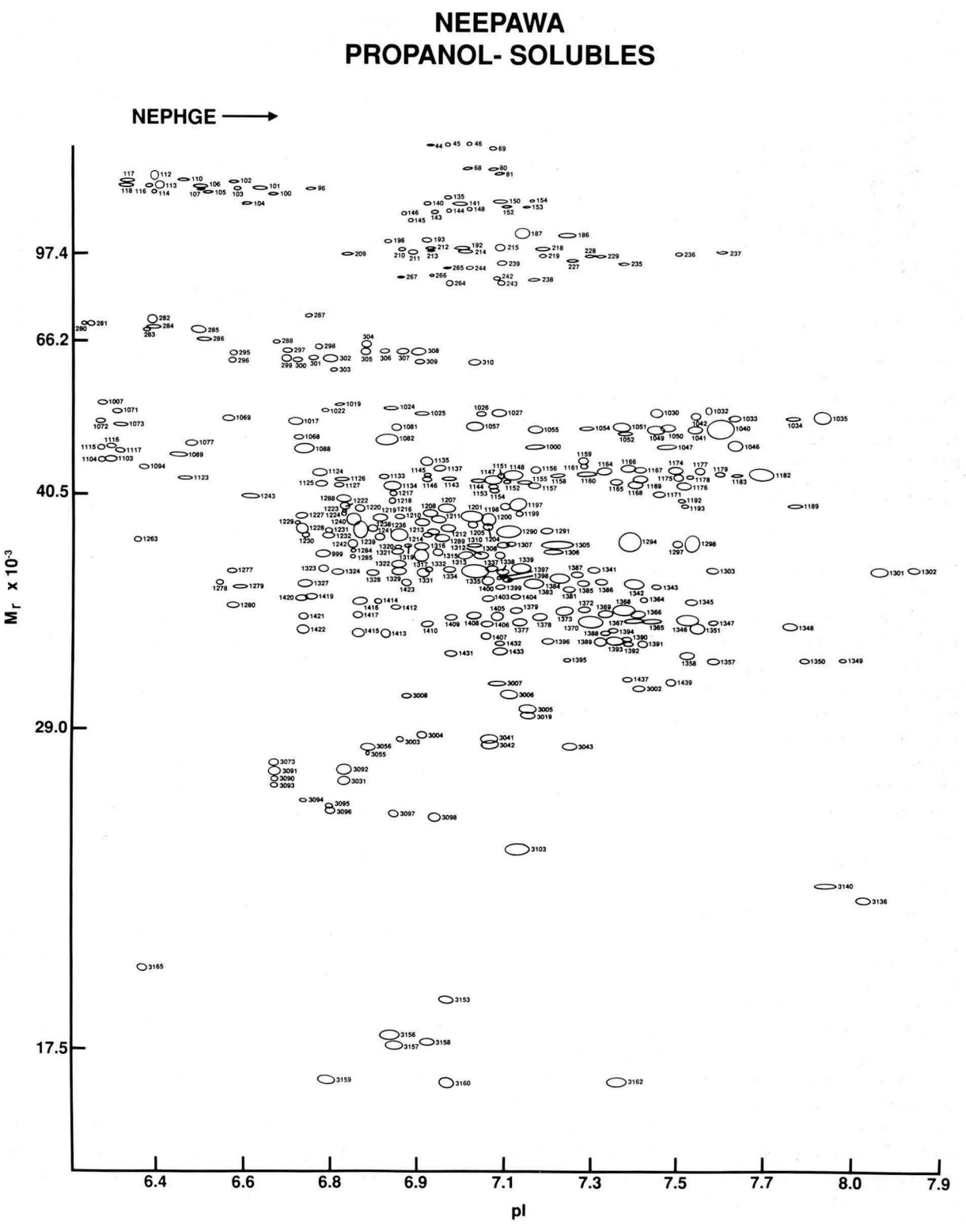

Fig. 7. Composite 2D (IEF + SDS-PAGE) separation of wheat prolamins. The first separation (IEF with pH 3–10 ampholytes; cathode at right) was followed by SDS-PAGE in a 15% gel. Data courtesy of R. Tkachuk.

The ability to achieve such separations is useless, however, without good data analysis. Image analysis can process 2D gel data [49,93]. Computer programs can normalize and correct captured gel images, giving spot files of position and intensity data which can identify varieties or indicate quality.

2.7. Capillary electrophoresis

Capillary electrophoresis is also a promising fractionation technique for proteins [94–97]. Separations occur in uncoated or coated glass capillaries (25–75 μm I.D.). High voltage (10–30 kV) plus efficient cooling permit rapid (10–30 min) high-resolution separations. Instruments are automated, give reproducible separations, and have good data capabilities. Fig. 8 shows a capillary electrophoresis separation of wheat proteins. Approximately 58 components result, counting partially resolved shoulders. This equals or exceeds resolution of most other one-dimensional methods. Capillary electrophoresis could become valuable for varietal identification, classification, and determination of quality.

3. CHROMATOGRAPHIC METHODS

Liquid chromatography is the second major method used to isolate and characterize wheat proteins [4]. Separations may be based on size or on characteristics imparted by specific amino acids. In size-exclusion chromatography, solutes move through columns packed with porous matrices at rates determined by relative sizes of solutes and matrix pores. In ion-exchange chromatography (IEC), solutes partition between the mobile phase and the support, to which they bind through ionized amino acids; mobility depends on strength of ionic interaction. In hydrophobic interaction chromatography (HIC), separations result from binding through non-polar amino acids to a lipophilic stationary phase.

Original chromatographic separations used large, hand packed columns. Resolution was sometimes good, especially for preparative purposes. As a rule, however, these methods are slow, labor intensive, and irreproducible, and results are hard to quantify. There have been few recent developments in such techniques. There have, however, been major improvements in high-performance liquid chromatography (HPLC) columns and instruments, and HPLC has become the method of choice for many applications. The following sections will first review the use of both types of chromatographic methods for wheat protein analysis.

3.1. Size-exclusion chromatography

Size-exclusion chromatography has indicated molecular sizes of native wheat proteins, shown

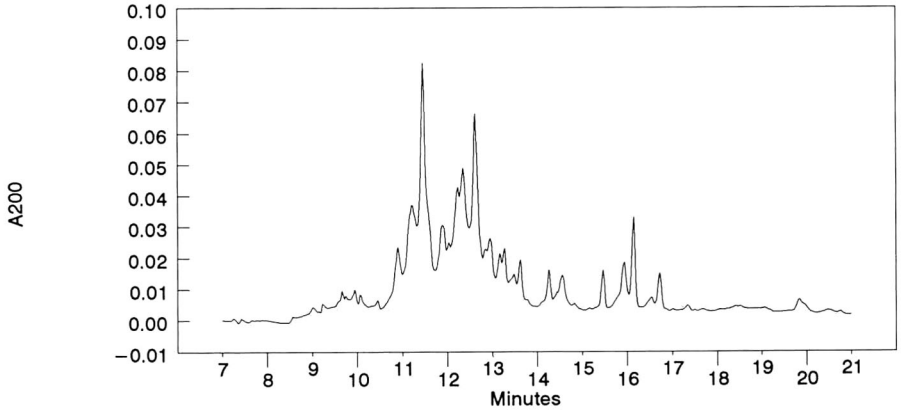

Fig. 8. Fractionation of wheat proteins by capillary electrophoresis. Wheat flour (cv. Centurk) was extracted with 30% ethanol, and the clear supernatant was separated at 10 kV and 40°C on a 75 μm I.D. uncoated capillary (40 cm from inlet to detector) in 0.06 *M* borate buffer, pH 9.0, containing 20% acetonitrile and 1% SDS. Proteins were detected at 200 nm.

how they are related, and revealed their subunit compositions. Gliadin, extracted with 70% ethanol, was first fractionated on Sephadex columns with dilute acetic acid as mobile phase into (a) an HMW (M_r 100 000–400 000) oligomeric fraction; (b) ω-gliadins (M_r 60 000–80 000); (c) γ-gliadins (M_r *ca.* 40 000); (d) α/β-gliadins (M_r 30 000–35 000); and (e) albumins plus globulins [98,99]. Similarly, using 4 *M* urea plus 0.03 *M* acetic acid as solvent, Huebner and Wall [100] showed that glutenin, after cleavage of disulfide bonds and alkylation of resulting cysteines, has three types of subunits differing in size and associative tendencies.

In subsequent size-exclusion chromatography studies [101–107], other solvents and more porous colums were used. Native glutenin was extracted with acetic acid plus urea plus cetyltrimethylammonium bromide [101,102], 5.5 *M* guanidine hydrochloride [104] or SDS [106,107], and fractionated by size on agarose supports. These studies showed size and compositional heterogeneity of native glutenin. M_r values were estimated at 5–20 million based on elution at column void volume or calibration with non-protein standards. Such values are thus questionable; the true M_r of glutenin remains unknown.

3.2. Ion-exchange chromatography

IEC has revealed much about gluten's composition. Woychik *et al.* [108] used carboxymethyl cellulose and a gradient of increasing acid concentration to fractionate gluten. Simmonds and Winzor [109] eluted proteins with a sodium chloride gradient in a 1 *M* dimethylformamide buffer. A useful fractionation was realized, but fractions were still heterogeneous.

IEC methods continued to improve by using dissociating agents (such as urea and dimethylformamide), adjusting elution conditions, and changing pH and ion-exchange media [110–113]. IEC on sulfoethyl cellulose, with a buffer of 3 *M* dimethylformamide, 1 *M* urea, 0.03 *M* acetic acid, and 0.005 *M* HCl and a guanidine hydrochloride gradient, first purified many glutenin subunits [100]. Such methods are still useful for preparative purposes [114–116], but are otherwise little used.

3.3. Hydrophobic interaction chromatography

HIC has been little used with gluten [117–121], largely because its resolution is limited. Yet, since HIC separates proteins on the basis of a unique complementary characteristic, surface hydrophobicity, and since it is the forerunner of reversed-phase (RP)-HPLC (see below), its use will be briefly reviewed.

Caldwell [118] showed that gliadins bind strongly, through hydrophobic sites on protein surfaces, to agarose having phenyl or octyl groups covalently bound. Gliadins were eluted with a gradient of increasing ethanol concentration. PAGE of fractions showed distinct differences: gliadins eluted in the general order ω, β, α and γ. Results showed the potential of hydrophobic separation methods for gluten fractionation.

Popineau and Pineau [120] used HIC, combined with IEC, to purify gliadins. A mixture of γ-gliadins was fractionated by HIC on phenyl Sepharose CL-4B. Purified subfractions were obtained in quantities sufficient for further characterization. Popineau [121] also showed that HMW glutenins could be fractionated by HIC. Glutenin fractions covered a wide range of hydrophobicity, and differed in subunit composition. HIC showed that hydrophobicity is an intrinsic property of gluten proteins.

3.4. High-performance liquid chromatography

While the above-described modes of chromatography can isolate and characterize wheat proteins, these methods are difficult and have many problems. They are slow, and labor intensive. Reproducibility is poor, and columns unstable. Resolution is often inadequate, and quantitation is difficult. HPLC columns and equipment overcame many of these deficiencies. In particular, introduction of uniform (*e.g.*, 5–10 μm) wide pore (typically >300 Å) silica packings with silanols derivatized to permit specific interactions and end-capped to prevent non-specific adsorption was a milestone [122–124]. Reliable equipment also became available with precise flow-rates and gradients, high reproducibility, sensitive detection, automatic operation and excellent data handling. These developments revolutionized isolation, characterization, and knowledge of wheat proteins [3–8,125–129]. The following sec-

tions review milestones in development and application of HPLC to wheat proteins, and describe recent progress.

3.4.1. High-performance size-exclusion liquid chromatography

High-performance size-exclusion chromatography (HPSEC) of wheat proteins was first described by Bietz [3,125], who used TSK-type columns with neutral phosphate–0.1% SDS buffers. Separations were rapid (about 20 min), sensitive, and reproducible. Quantitation was achieved at 210 nm, and indicated M_r agreed well with those from other techniques. Resolution was as good as or slightly better than that by open-column size-exclusion chromatography.

Results also showed that HPSEC can differentiate varieties by analyzing native glutenin or total proteins, and can predict breadmaking quality from M_r distributions of native glutenin or its reduced subunits [130]. These studies showed, however, that reproducible protein extraction is a problem, and that protein M_r distributions change with time due to association.

These problems were dealt with by Dachkevitch and Autran [131], who extracted unreduced flour proteins for 2 h at 60°C with 0.1 *M* sodium phosphate, pH 6.9, containing 2% SDS. The centrifuged extract was analyzed on a TSK 4000SW column. Proteins were extracted reproducibly though not quantitatively; extracts were stable for at least two days. When applied to wheats from different locations and years, this procedure yielded size distribution data highly correlated with baking quality. Results prove the value of HPSEC of unreduced wheat proteins in breeding.

Singh and co-workers [132,133] described another way to extract unreduced wheat proteins for HPSEC. Flour was briefly sonicated in 0.5 *M* sodium phosphate, pH 6.9, containing 2% SDS, gently agitated for 30–120 min, and centrifuged. Extraction was complete without addition of a disulfide bond reducing agent. Proteins from strong wheats were more difficult to extract. This procedure apparently disrupts major non-covalent and covalent forces joining polypeptides. Shear degradation of disulfides probably occurs, converting insoluble HMW glutenin into lower-M_r soluble species.

Proteins extracted by this method from wheats varying in breadmaking quality were analyzed by HPSEC [133]. Three peaks resulted, corresponding to glutenin, gliadin, and albumins/globulins (Fig. 9A). Areas of peaks correlated significantly with breadmaking quality: percentage of the first peak (polymeric glutenin) was highly positively correlated with loaf volume, dough resistance and extensibility, and dough development time. Structural features that differentiate strong and weak wheats must still be retained after sonication. This procedure can be a rapid small-scale (*e.g.*, half-kernel) test for predicting quality potential during breeding.

The low resolution of most HPSEC separations of wheat proteins reflects their size heterogeneity. Batey *et al.* [134] also found, using this procedure, a gradual deterioration in column performance, attributed to SDS in the buffer. The normal buffer was thus replaced with 50% acetonitrile containing 0.1% trifluoroacetic acid. This solvent stabilized the column, and significantly enhanced resolution (Fig. 9B), presumably by disrupting hydrophobic forces which prevent complete dissociation of proteins.

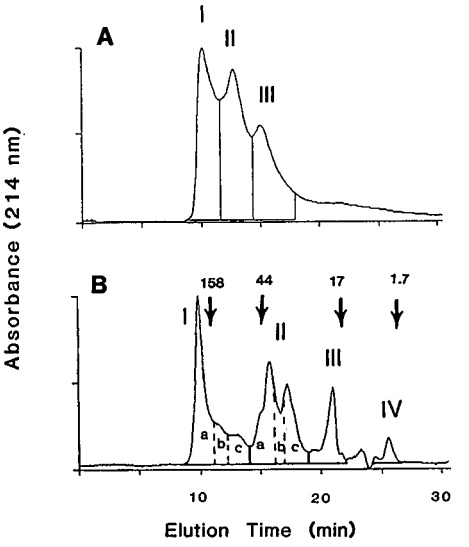

Fig. 9. HPSEC separations of wheat proteins from the cultivar Cook on a Waters Protein Pak 300 column. Proteins were extracted with sonication using 0.05 *M* phosphate buffer, pH 6.9, containing 2% SDS, and analyzed (A) using 0.05 *M* phosphate, pH 6.9, containing 0.1% SDS as chromatographic buffer and (B) using a buffer of 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The numbers at the arrows in (B) indicate $M_r \times 10^{-3}$. I–IV and a–c represent specific fractions correlated with quality. From Batey *et al.* [134].

Other extraction and solvent conditions may also be useful. Huebner *et al.* [135] extracted flour with 70% ethanol to solubilize gliadins, and 6 *M* urea–0.05 *M* sodium phosphate, pH 7.5–0.3% dithiothreitol–1% SDS to solubilize glutenin subunits. HPSEC on a Pharmacia Superose 12 column using 0.1 *M* sodium phosphate, pH 7.1, containing 20% acetonitrile, 0.3% SDS, and 0.1% dithiothreitol revealed amounts of HMW gliadins, ω-gliadins, LMW gliadins, albumins, and HMW and LMW glutenin subunits. Statistical analyses of data accurately classified hard red spring and winter wheat cultivars, which has been difficult or impossible by most methods.

3.4.2. High-performance ion-exchange liquid chromatography

As noted above, IEC on traditional columns gives good separations of gluten. This suggested that high-performance IEC (HPIEC) should give especially good separations of these proteins. Surprisingly, however, only two publications report HPIEC separations of gluten polypeptides.

Batey [136] first fractionated gliadins by anion-exchange HPLC on Pharmacia Mono-Q. Only under very alkaline conditions (*e.g.*, pH 10.4), where arginine's ionization is suppressed, were good separations possible. Wheat varieties could be identified, but far fewer gliadins resolved than by PAGE or open-column cation-exchange chromatography.

Most IEC separations of gliadins have been on cation-exchange media, where molecules are positively charged. Larre *et al.* [137] thus attempted cation-exchange HPIEC of gliadins on Pharmacia Mono-S HR. About 20 peaks resolved during 110 min, fewer than by PAGE or RP-HPLC. Each peak consisted of a well resolved group of proteins. Cultivars could be differentiated, showing potential of cation-exchange fast protein liquid chromatography (FPLC) for varietal identification. It remains a challenge, however, to achieve high-resolution HPIEC separations of gluten proteins.

3.4.3. Reversed-phase high-performance liquid chromatography

Adoption of RP-HPLC as a fractionation technique for wheat proteins has been a major development during the last decade. RP-HPLC separates proteins based on surface hydrophobicity, as in a few previous HIC studies.

During the 1970s, RP-HPLC came into wide use for LMW solutes. Columns contained uniform porous silica microspheres (10–30 μm diameter), converted to reversed-phase columns by derivatizing silanols with hydrophobic (*e.g.*, C_{18}, C_8 or phenyl) ligands. Some free silanol groups remained, however, which could ionically bind proteins. Packings typically had 80–100 Å pores, too small to allow ready access of most proteins. Thus, most attempts to use these materials for proteins failed.

Better HPLC columns overcame these deficiencies [122–124]. Wide-pore silicas were derivatized with hydrophobic ligands, and residual silanols were end-capped. Superior equipment also became available.

These developments permitted excellent separations of proteins, and were first applied to gluten by Bietz [138]. Several papers have reviewed RP-HPLC studies of wheat and other cereal proteins [3–8,125–129]. This section will briefly review RP-HPLC achievements and applications, and describe several recent studies.

Methods first described for wheat protein RP-HPLC [138] are still applicable. Proteins are extracted with nearly any solvent, and fractionated on C_4, C_8 or C_{18} columns (150–250 cm long × 4–5 mm I.D.) using a gradient of increasing acetonitrile content (typically between 20% and 60%) with 0.05–0.1% trifluoroacetic acid. Detection is usually at 210–225 nm. Constant temperature ensures good reproducibility, and elevated (50–70°C) temperature often enhances resolution by disrupting hydrogen bonds [139].

Marchylo and Kruger [140] discovered a precaution for wheat protein RP-HPLC. If too large a volume of hydrophobic sample is applied, some proteins, particularly those least hydrophobic, may not bind, and elute with the void volume peak. This can be prevented by limiting injection volume, using multiple small injections, or using less hydrophobic solvents.

3.4.4. Gluten fractionation and characterization by RP-HPLC

All types of wheat proteins have been analyzed by RP-HPLC by varying extraction conditions and gradients. Good separations of gliadins were achieved first [138]. As columns and our understanding of how to use them have improved, so

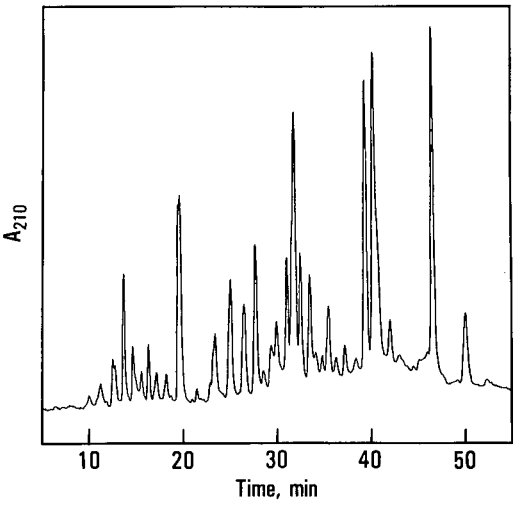

Fig. 10. RP-HPLC separation of gliadins extracted with 70% ethanol from the wheat variety Siouxland. A Vydac C_{18} column was used at 60°C, with a 27–45% acetonitrile (+0.1% trifluoroacetic acid) gradient during 50 min. From Bietz [5].

have separations (Fig. 10). Fifty or more peaks typically resolve from whole gliadin. This exceeds resolution of all except the best 2D PAGE methods.

RP-HPLC has other important advantages. Reproducibility is excellent: retention times typically vary by no more than a few hundredths of a minute. Over long periods, excellent reproducibility can be achieved by normalization based on periodic analysis of standards to compensate for slight changes in the column with time [141–143]. RP-HPLC is also fast; most separations take 30–120 min. If maximum resolution is not needed, smaller columns plus faster flow-rates and steeper gradients can give faster separations [139]; certainly narrow-bore columns will give even greater speed. RP-HPLC is also very sensitive. For example, in breeding a kernel can be cut in half, and analyses done on proteins from the brush end. The germ remains available for propagation if desired.

RP-HPLC's automated nature is another major advantage. An automatic sample injector, as part of a modern system, decreases operator time, increases number of samples analyzed, and improves reproducibility. HPLC is also more easily controlled than is gel electrophoresis. RP-HPLC data can also be accurately quantified. While some amino acid side chains contribute to protein absorbance at 210–225 nm, most absorbance is due to the peptide bond, making absorbance related to mass of protein. Thus, Sutton [144] related variation in amounts of HMW glutenin subunits, as measured by RP-HPLC, to baking performance and quality. The last major advantage of RP-HPLC is that it complements other methods. Proteins separate by surface hydrophobicity, not size or charge. Thus, RP-HPLC may show single PAGE or IEF bands to contain several polypeptides varying in hydrophobicity, and RP-HPLC peaks often contain several charge or size variants [145].

RP-HPLC also gives excellent separations of glutenin. These are more complicated than gliadin analysis since sequential extraction is needed to separate glutenin from other proteins. Disulfide bonds must also be cleaved to liberate glutenin subunits; resulting cysteines are often alkylated to prevent reoxidation. Fig. 11 shows a typical separation of reduced–alkylated glutenin subunits [146]. HMW subunits, associated with breadmaking quality, elute first, and are well resolved; LMW subunits elute last, and are not as well resolved. Better fractionation of these LMW subunits is possible if they

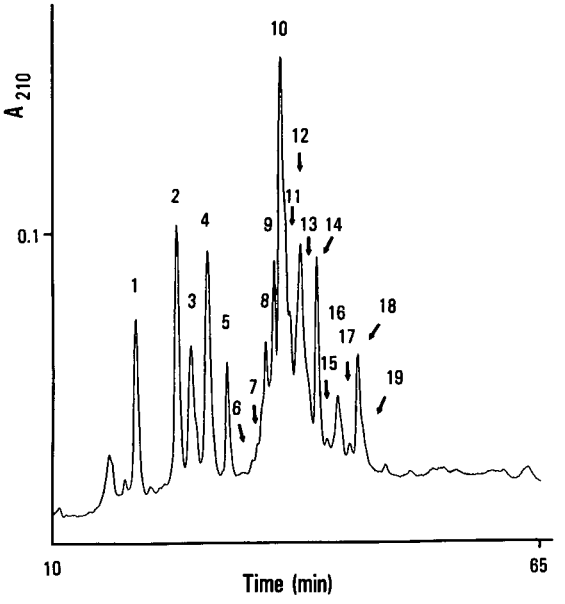

Fig. 11. RP-HPLC separation of pyridylethylated glutenin subunits from the wheat variety Chinese Spring. Peaks 1–4 correspond to the HMW subunits from this cultivar; later eluting polypeptides are LMW subunits. From Burnouf and Bietz [146].

are first separated from HMW subunits by solubility in neutral 70% ethanol [147]. LMW glutenin subunits then resolve into 20 or more components that differentiate varieties and indicate baking quality.

3.4.5. Varietal identification and genetic studies

As with PAGE, varietal identification from gliadin fingerprints is possible by RP-HPLC [148]. Expression of these proteins is nearly constant for a variety, though slight quantitative differences may occur for a variety grown at different locations [149–151]. HMW and LMW glutenin subunits can also differentiate and serve to identify cultivars [147].

RP-HPLC can also be useful during breeding to select germplasm based on specific proteins. For example, RP-HPLC can detect the 1BL/1RS wheat-rye translocation [152], which transfers desirable rye characteristics to wheat, but may also make dough sticky or reduce gluten strength. Similarly, Sutton *et al.* [153] showed that quantitative RP-HPLC of HMW glutenin subunits indicates potential loaf volume and bake scores in breeding lines.

RP-HPLC can also prove varietal purity. For example, the land race "Nap Hall", a source of genes for high protein and lysine, is very heterogeneous [148]. Heterogeneity also occurs, as biotypes, in many modern varieties. RP-HPLC and PAGE are equally effective in discriminating biotypes [154].

In the USA, wheat is traded by class, not variety. Some classes, such as hard red spring and winter, are difficult to differentiate. Endo *et al.* [155,156] differentiated these classes from integrated gliadin RP-HPLC data. The statistical partial least squares (PLS) procedure also correctly classified many hard red winter and spring varieties from non-integrated RP-HPLC gliadin data [157].

Scanlon *et al.* [143] showed that varietal identification can be made automatic and objective by gliadin RP-HPLC analysis. Normalized peak heights and retention times provide characteristic signatures that, through comparison to data for known wheats, reliably identify varieties.

Many wheat aneuploids, having absent or duplicated chromosomes or chromosome arms, are available. RP-HPLC of proteins from such lines can locate genes that code specific polypeptides. Gliadins and glutenin subunits were analyzed by this procedure [146,158]. Results identified wheat's HMW glutenin subunits, associated with breadmaking quality, and showed that each gluten polypeptide type has unique surface hydrophobicity characteristics.

3.4.6. Quality prediction by RP-HPLC

RP-HPLC can indicate quality by analyzing proteins which directly affect functionality, such as HMW glutenin subunits, shown by SDS-PAGE [63] and RP-HPCL [159] to be markers of alleles associated with breadmaking quality. Other proteins may be markers of genes linked to other genes which directly affect quality. For example, Bietz *et al.* [148] used RP-HPLC to show that late eluting γ-gliadins were correlated with pasta quality. These proteins correspond to gliadins "42" and "45", associated with durum weakness and strength, respectively [160]. This characteristic could be screened for by RP-HPLC in as little as 5 min.

Another example of RP-HPLC revealing wheat

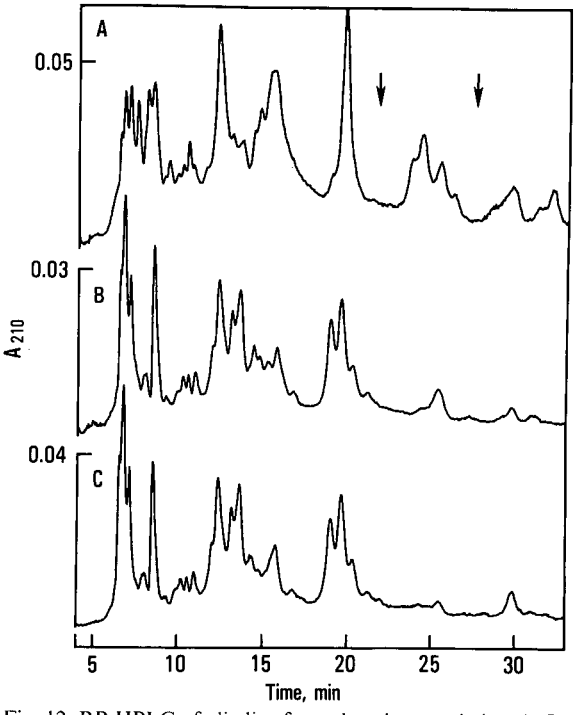

Fig. 12. RP-HPLC of gliadins from the wheat varieties (A) Iuanillo, (B) Westbred and (C) Yecora Rojo. The area of peaks eluting between the two arrows was negatively correlated with baking quality. From Huebner and Bietz [161].

quality is in Fig. 12. Comparison of integrated data with baking characteristics enabled Huebner and Bietz [161] to identify a late eluting baking quality gliadin fraction, the area of which correlated negatively with quality measurements. Selection for this criterion could be useful for breeding, marketing, and quality control.

Another interesting study relating gluten to breadmaking quality is by Van Lonkhuijsen *et al.* [162]. HMW glutenin subunits explain only part of the variation in wheat quality, so wheats with the same HMW glutenin subunits, but varying in quality, were studied. Gliadins were fractionated by RP-HPLC, and data were integrated and analyzed statistically. Results identified specific gliadins which strongly influence breadmaking properties.

Others are also using RP-HPLC data to study quality. Primard *et al.* [163] showed, by statistical analysis of RP-HPLC and SDS-PAGE data, that breadmaking quality depends on many different proteins. The dimension reduction techniques PLS and principal component analysis (PCA) can also objectively identify quality related proteins without integrating RP-HPLC data [157,164].

3.4.7. Role of computers in wheat protein RP-HPLC

Today, chromatographic software is available from HPLC suppliers and other sources that permits acquisition and storage of raw, non-integrated data for later processing. Ten years ago, however, this was not true. Many of the first HPLC studies of wheat proteins at the National Center for Agricultural Utilization Research, Peoria, IL, USA, were possible only because R. Butterfield and colleagues of the computer staff developed programs that permit unattended acquisition of raw data (*i.e.*, detector readings at equal intervals) and its storage on a mainframe computer. Stored data could then be integrated automatically, or in a manual mode in which the operator specifies peaks and baselines, or even by Gaussian deconvolution. Baselines could be corrected. Data could be viewed many ways to compare chromatograms, and plotted to any scale. Chromatograms could be directly compared, permitting aneuploid analysis, mixture analysis, and determination of pedigrees. Data could also be translated to a form used by statistical programs to relate protein composition to quality.

4. INTERPRETATION OF ELECTROPHORESIS AND CHROMATOGRAPHY DATA

The mobility–density plot commonly reported in electrophoresis is a spectrum of bands with varying intensities and locations, which characterizes the solute. Earlier studies focused on interpretation of these spectra, primarily in terms of presence or absence of certain bands, but also incorporating ordinal intensity scores to refine characterization of the solute (see, for instance, ref. 165). More recently, densitometry provides a continuous trace of density *vs.* mobility. The resulting plot has the same character as a chromatogram. As a result, similar statistical methods apply to both electrophoresis and chromatography. This discussion treats both methods at once, because the basic statistical object is the same in each case: a plot of intensity *vs.* location (mobility for electrophoresis; retention time for chromatography). The major choices to be made in treating the data are (a) whether to normalize the area under the plot or the location; (b) whether to operate on peak areas or heights; and (c) whether to interpret the intensity *vs.* location plot directly.

4.1. Subjective visual methods

Damidaux *et al.* [166] found, by inspection of electropherograms, that durum wheats having gliadin "42" (indicating relative mobility) tend to have weak pasta quality, but those having instead band "45" tend to have strong pasta quality. Burnouf and Bietz [160] observed precisely analogous peaks by RP-HPLC of durum wheats, again based on subjective visual inspection of data. The dinstinction is so clear that one hardly needs statistics. Fig. 13 shows the componentwise mean chromatograms from RP-HPLC of a collection of group 42 and group 45 durum wheats. The graph also shows componentwise standard deviations for variation about the mean in each group.

In more complex examples, however, formal statistical methods come to the fore. The eye may see patterns simply because of random fluctuations. In such instances a test of statistical significance is needed. On the other hand, strong trends involving multiple variables may be masked in visual inspection of the data. In such instances multivariate statistical methods are often useful.

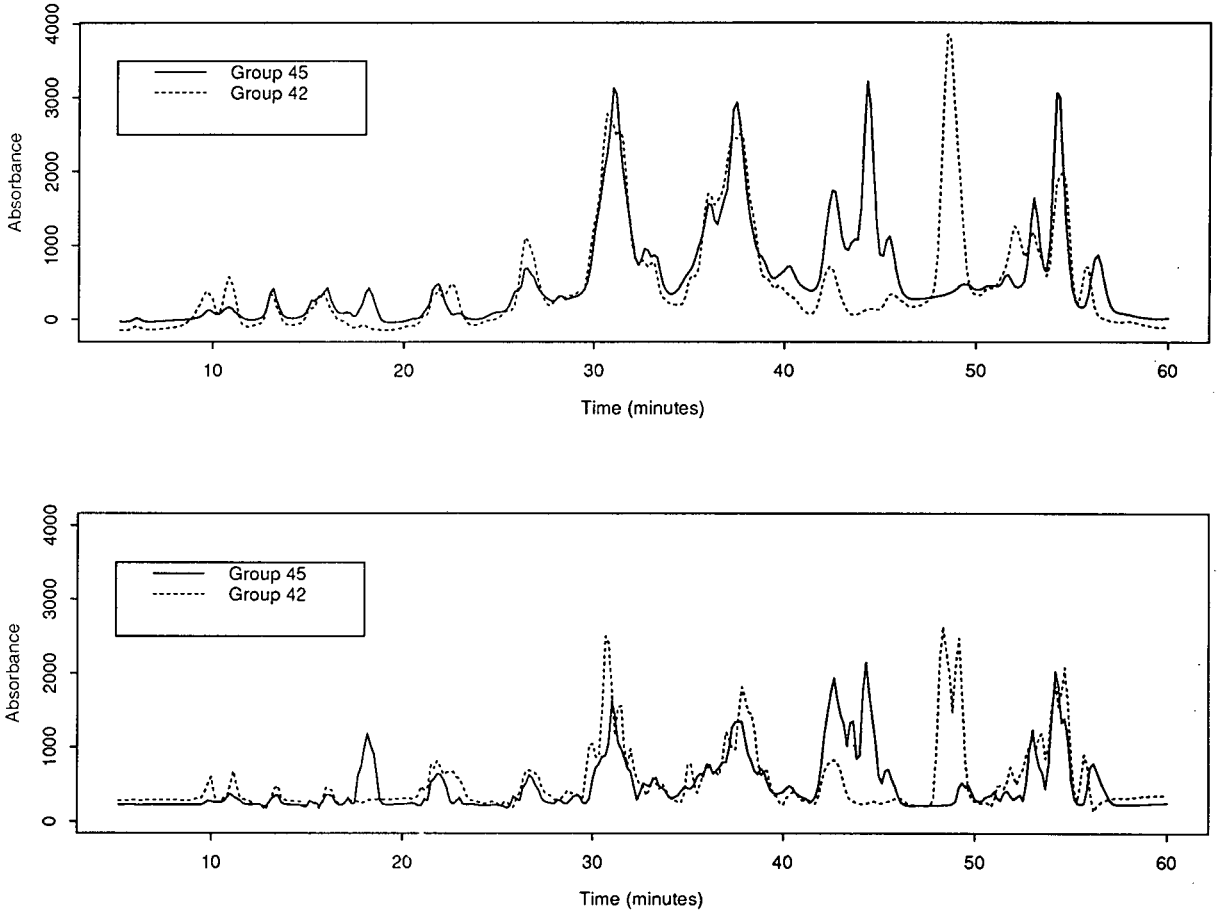

Fig. 13. Componentwise means (top) and standard deviations (bottom) for group 42 and group 45 durum wheats.

Statistical methods often have corresponding plots that can enhance understanding of the data. For instance, in multiple linear regression the effect of a variable can be seen from the so-called added variable plot. This is a scatter plot of projections of the response and regression variable into the space orthogonal to the remainder of the regression variables in the model [167]. The slope of a least squares line fit to this scatter plot is equal to the least square estimate of the parameter for this variable for the multiple linear regression model. Such plots can reveal violations of the modeling assumptions such as non-linearity.

It is worth emphasizing therefore that summary statistics and computational algorithms do not replace visual inspection of data. Graphical methods may reveal clear patterns in data that render unnecessary the need for formal statistical methods. On the other hand, preliminary plots of data may expose problems that affect the type of analysis to be done. Texts on applied statistics such as Weisberg [167] emphasize the use of graphical diagnostics such as residual plots, which can reveal unanticipated phenomena and point to important refinements of the model.

4.2. Varietal identification via similarity scores

The earliest attempts at quantitative analysis of electrophoresis data were concerned with identification of wheat varieties by comparison with a library of electrophoregrams of known varieties [43,165].

An electropherogram to be classified would be scored on the basis of peak matching to each member of the library. Usually one or more reference varieties would be run on the same gel to standardize relative mobilities and peak or band intensities. Lookhart *et al.* [165] used a five-point scale for intensity, and matching scores for the agreement between each pair of peaks. The overall agreement score was obtained by summing over all the peaks. The new sample would then be classified according to the highest match score in the library. Sapirstein and Bushuk [43] used a related strategy, but with a nine point scale for intensity and a different set of scores for matching. In both cases intensities were determined subjectively. More recent work used densitometry to automate scoring of intensities. For instance, Cox *et al.* [168] scanned electropherograms on a densitometer and discretized the output to a scale like that of Lookhart *et al.* [165].

If a comprehensive library of types is available, similarity scoring is appealing in its directness. However, these may be room for improvement in the methodology. Little guidance is available on how to decide if a previously unclassified variety has been encountered. Empirical guidelines might be developed on the basis of cumulative experience, but these would be highly context dependent, depending on the current library of types and presumably on the type of grain under study. There is no mathematical reason to restrict to ordinal scores in comparing peak patterns. One might just as easily measure similarity or distance between vectors of densities or peak heights. The main practical considerations in the use of oridinal scores seem to be that they are less susceptible to anomalous readings than raw peak heights, and they may be easier to scale.

4.3. Hierarchical clustering methods

One method of enhancing the analysis is to use similarity scores to cluster varieties into more homogeneous subgroups, and then to characterize the groups in terms of their physical properties. This was the approach of Wrigley *et al.* [169] and Du Cros [170] who employed the minimum spanning three (MST) to develop empirical taxonomies of wheat varieties from similarity scores. The resulting tree-structured organization of the varieties provided the means for grouping them. An added feature was the graphical representation of degree of similarity in terms of distances along branches of the tree. Gower and Ross [171] provided a clear exposition of the method.

Similarities are generally expressed as fractions or percentages. Assuming they are fractions between zero and one they may be translated into distance-like measures in the following way:

$$\text{distance}^2 = 1 - \text{similarity}.$$

Other schemes are possible, and it is often easier to start with a distance rather than a similarity measure. There are mathematical and algorithmic benefits in choosing the distance measure to be a true distance in the sense of obeying the axioms of Euclidean geometry [171].

It is perhaps easiest to understand the MST in the two-dimensional case. Fig. 14 shows the MST for 17 wheat varieties based on the heights of two RP-HPLC peaks, selected only to obtain a somewhat complex MST for illustration. The MST was computed with the aid of S-Plus (Statistical Sciences, Seattle, WA, USA). Pairs of peak heights are represented as points in the plane. They are joined by line segments to form a tree. The MST shown is the tree whose branches have minimum total length among all possible spanning trees. Formally a spanning tree is a structure in which: (1) there are no closed loops; (2) each point has at least one line segment attached to it, and (3) there is a path from any point to any other point [171]. The two-dimensional case is easiest to understand because distances between all points are represented by distances in the plane. With more than two variables a plot like Fig. 14 will accurately reflect distances between neighboring points on the tree, but it will give little information on distances between nonadjacent points. Friedman and Rafsky [172] discussed the use of the MST for plotting multivariate data.

The MST contains the information needed to construct a single linkage clustering tree as illustrated in Fig. 15. Single linkage means that two clusters are joined at level h if at least one pair of points, with one member of the pair from each cluster, is within h units. The horizontal scale in Fig. 15 is the largest distance from any point to its nearest neighbor in the cluster. Thus, if we require each point in a

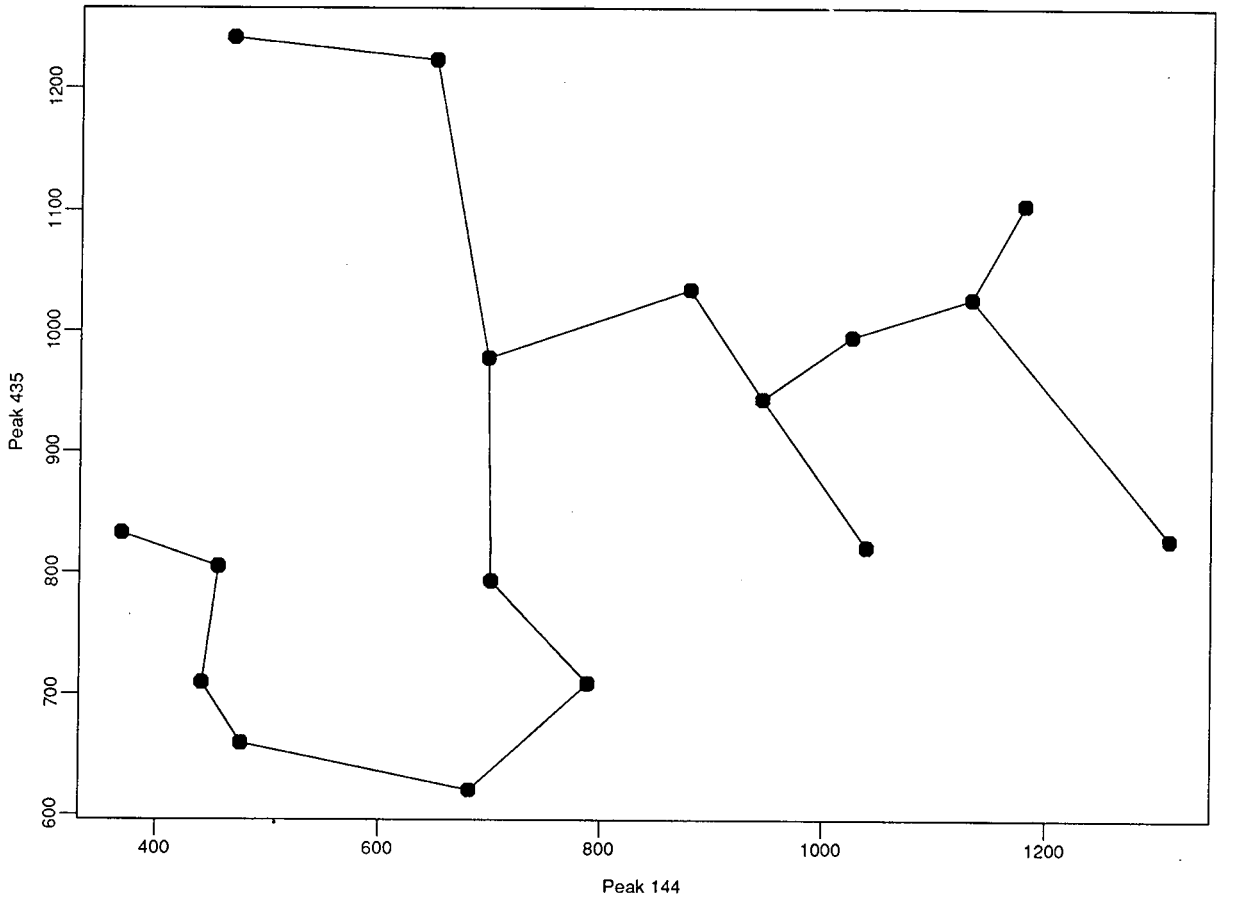

Fig. 14. Minimum spanning tree for 17 wheat samples, based on two minor HPLC peaks.

cluster to be within 200 units of its nearest neighbor we obtain four clusters: a singlet containing only variety 11, a doublet containing varieties 6 and 7, a four-point cluster of varieties 1–4, and a large cluster of all other varieties in the sample.

Wrigley [173] discussed a subtle issue of interpretation in relating clusters to baking quality due to the observational nature of the data. Varieties in the same cluster have similar electrophoresis patterns and may have similar pedigrees as well. Because of the observational nature of the data, it is possible that any association between the clustering and baking properties is due to other properties associated with the pedigrees of the wheats rather than features reflected by electrophoresis. With these kinds of studies one hopes to see a confirmation (or refutation) of initial results as more data are compiled.

The single linkage clustering tree is an empirical taxonomy of varieties. Different choices of peaks may produce very different trees, and one would hope to achieve some stability by including more peaks. Further work is needed to determine appropriate distance measures for the types of data seen in wheat studies. A promising approach was discussed by Marshall *et al.* [174] in analyzing HPLC chromatograms of urine proteins in which each chromatogram is treated as a continuous signal. In defining an L_2 type distance they incorporated time-dependent location shift functions, to account for misalignment of peaks, and time-dependent weights. Alignment is also an issue in wheat studies.

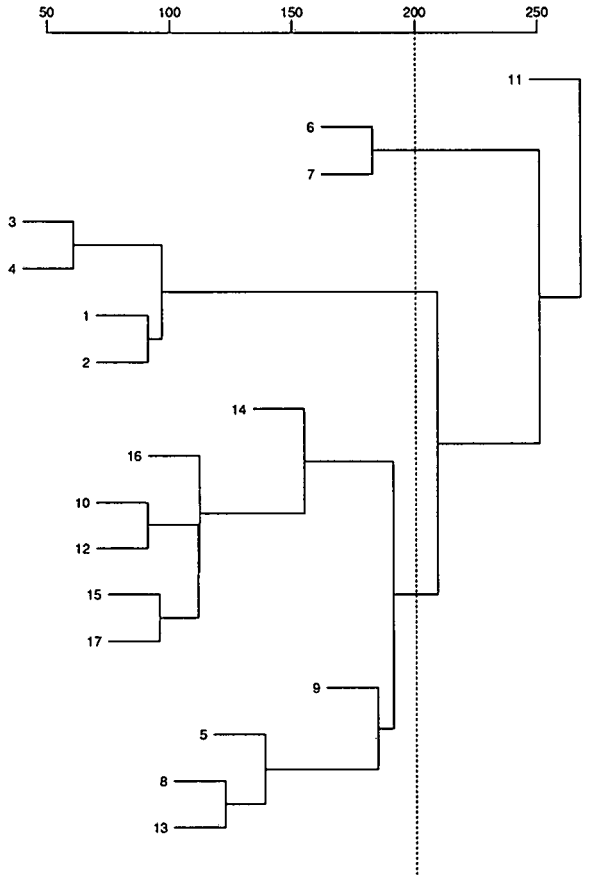

Fig. 15. Single linkage clustering tree for 17 wheat samples. based on two minor HPLC peaks.

Column performance can vary over time, causing shifts in retention times. Sapirstein *et al.* [141] discussed a piecewise linear location shift method using several reference peaks from a concurrent standard chromatogram.

Despite these methodological questions, single linkage clustering can separate known groups such as durum wheats, and the MST appears to be a useful tool for plotting multivariate data.

4.4. Principal component methods

PCA has a history dating at least to Pearson's 1901 article [175] "On lines and planes of closest fit to systems of points in space". The idea is to project multivariate data onto lower dimensional planes that are closest on average to the points in the higher dimensional space. At the first step, all points are projected onto a line selected to be as close as possible, on average, to points in the multivariate data set. It turns out to be equivalent to projecting the points on a line selected to maximize the sample variance of the projections along the line [176]. One then selects a second line, constrained to be orthogonal to the first, such that sample variance along the line is maximized. Then a third line orthogonal to the first two lines is selected, again maximizing sample variance. The process may repeat until the number of axes is equal to the smaller of the number of objects and the number of variables. In fact, basis vectors along the PCA axes and the corresponding variances are usually computed all at once, either by a singular value decomposition of the data matrix or by a spectral decomposition of the sample covariance matrix [177].

PCA is sensitive to scaling of different variables, so it is common to standardize variables first, subtracting the componentwise sample mean and dividing by the componentwise standard deviation. This is equivalent to replacing the sample covariance matrix by the sample correlation matrix in the analysis.

Often the first few components of the rotated data contain most of the variation. Scatter plots of these components can be useful for detecting clusters, patterns and outliers in the data. For instance, Fig. 16 is a scatter plot of the first two components from PCA of 112 durum wheats [178]. The raw data contain nine quality measurements of each sample. The scatter plot shows how group 42 and group 45 samples separate in the plane defined by the first two rotated components, indicating substantial differences in quality. Interestingly, the best separating plane is diagonal to the axes, and neither component by itself would be effective for separating the groups.

The basis vectors themselves are of interest because they indicate which variables account for the most variation. Fig. 17 shows superimposed RP-HPLC chromatograms for 12 varieties of hard red spring wheat. Fig. 18 shows the first two basis vectors determined by PCA of the vectors obtained by sampling the chromatograms every 5 s [164]. The first basis vector is primarily a difference across a set of highly variable peaks with retention times be-

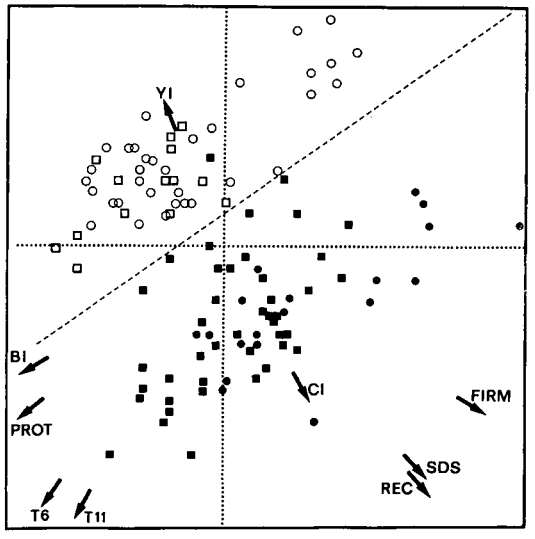

Fig. 16. First two components from PCA of 112 durum wheat samples using 9 quality tests: spaghetti yellow index (YI), spaghetti brown index (BI), protein content (PROT), minimum cooking time + 6 min (T6), minimum cooking time + 11 min (T11), microdisks cooking index (CI), gluten elastic recovery (REC), sedimentation volume (SED), and gluten firmness (FIRM). Open squares are type 42 winter wheats; open circles are type 42 spring wheats; solid squares are type 45 winter wheats, solid circles are type 45 spring wheats. From Autran *et al.* [178].

tween 27 and 28 min. The second basis vector indicates where the variation orthogonal to the first basis vector occurs. It weights on various retention times with no single dominant site. Note that PCA does not require peak identification; it can be applied directly to the discretized curves.

Autran *et al.* [178] made clever use of PCA in connection with correlation analysis of quality measurements. Autran and Galterio [179] used a similar method to study correlations among electrophoresis bands. Both articles provided correlation matrices and pairwise tests for zero correlation among the variables. Care is needed in interpreting significance tests, because multiple testing on the same data increases the experimentwise false positive rate, often drastically. Plotting the first two components from PCA on the correlation matrices as in Fig. 16 provided useful insight into clumping and patterns in the data.

PCA is commonly used with regression analysis when there are many potential regression variables. Principal component regression is a composite methodology in which the response variable is regressed on principal component projections of the regression variables [176]. The constructed regression variables are mutually orthogonal, which implies that the corresponding regression parameter estimates are uncorrelated and simplifies the interpretation. Jolliffe [176] and Martens and Naes [180] discussed variable selection in principal component regression, the point being that components accounting for the most variation among the regression variables need not be the best predictors of the response variable.

4.5. Multiple linear regression and peak selection

Various researchers have used linear regression analysis to model the dependence of wheat quality

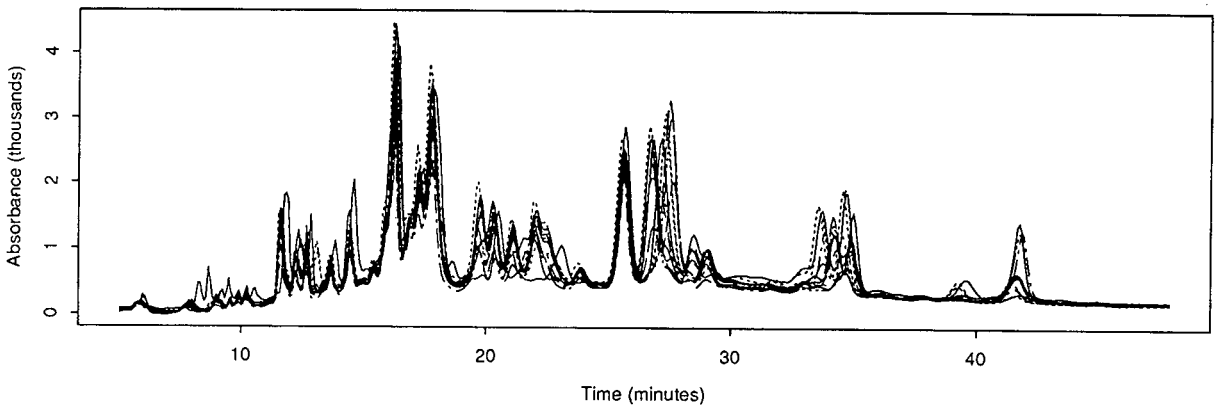

Fig. 17. RP-HPLC chromatograms for 12 varieties of hard red spring wheat. From Simpson *et al.* [164].

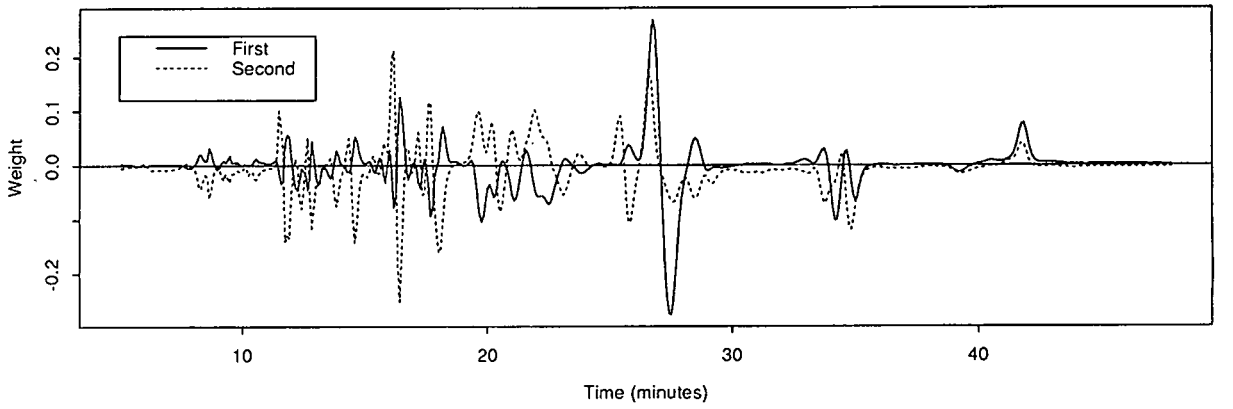

Fig. 18. First two basis vectors from PCA of 12 varieties of hard red spring wheat. From Simpson *et al.* [164]

on bands or peaks from electrophoresis and chromatography. Considerable effort goes into the identification and standardization of peaks, and different methods have been used. Ng and Bushuk [181] defined binary regression variables indicating presence or absence of specific glutenin electrophoretic bands. Then they used stepwise regression to select variables to include for linear prediction of a baking strength index. Scanlon *et al.* [182] defined quantitative regression variables by determining areas under consecutive regions of RP-HPLC chromatograms. They used stepwise variable selection to develop a linear regression model for predicting dough extensibility. Careful treatment of retention times is necessary, and some kind of normalization appears helpful. They noted that some regions may contain more than one peak, which might cause some effects to be masked. Primard *et al.* [163] and Van Lonkhuijsen *et al.* [162] used related approaches. Automating peak identification for regression analysis appears to be a challenging problem.

Parameter estimates from these studies are often poorly determined despite apparent statistical significance. In a thorough investigation Scanlon *et al.* [182] observed sign changes in parameter estimates and quite different sets of predictor variables between two replications of stepwise variable selection. This illustrates one difficulty in interpreting models found by variable selection procedures: the statistical significance of t-tests after variable selection can be grossly optimistic and should be given little credence. There is, for instance, an example due to Freedman [183] in which independent standard normal random variables generated by computer were used as responses and potential predictors in stepwise regression. Despite the independence of all the observations, a model was found that included several variables having significant t-tests and a high value of R^2, the coefficient of determination.

Another reason for poorly determined parameter estimates might be the parametrization. Suppose that the peak identification problem has been solved and the chromatogram or electrophoregram has been subdivided into peaks and normalized so that total area is 100%. Suppose p peaks are included in the model, and their relative areas are X_1, $X_2, \ldots, X_p$. The usual way to parameterize the linear regression model is to write

$$Y = a + b_1X_1 + b_2X_2 + \ldots + b_pX_p + e$$

where $a, b_1, \ldots, b_p$ are regression parameters and e is random with mean zero and unknown variance. Here the slope estimates are incremental effects of specific peaks relative to all other peaks that have been left out of the model. Thus the interpretation of all the regression parameters changes if we remove or add a peak to the regression model. One way around this problem is to reparameterize the model. Since the total area is 100%, we can replace the constant (intercept) by the constructed variable

$$X_0 = 100 - X_1 - X_2 - \ldots - X_p$$

to obtain the equivalent model

$$Y = b_0X_0 + b_1X_1 + \ldots + b_pX_p + e$$

In the latter expression the parameters reflect the degree to which the response is due to different peaks regardless of which other peaks are in the model. The two forms of the linear model are equivalent in that they yield identical predictions and sums of squared residuals, but parameters in the second form may be more stable when the chromatogram is normalized. If the stepwise search is used the change in parametrization should be done after variable selection, because X_0 cannot be selected independently of other variables in the model. Simpson *et al.* [184] used this parametrization to improve interpretation of regression parameters in an example involving attribution of pollution in rivers to different uses of the surrounding land, a scientifically distinct but statistically related problem.

The predictions in multiple linear regression are usually better determined than the individual parameter estimates. The main concern in the case of prediction is to avoid extrapolating outside the range of regression variables used to develop the model. Non-linearity and other problems with the model can easily crop up outside the range of experimentation where little information is available on the nature of the response [167].

4.6. Partial least squares

PLS is a relatively new method often used in chemometric applications when there are many possible variables and a method for predicting or calibrating is desired [185,186]. The method has features in common with PCA and regression analysis and is meant to handle problems with multiple predictors and responses, as well as simpler problems with one response and many predictors. The latter case is easiest to understand.

Suppose we wish to relate the response Y to regression variables $X1$, $X2$, ..., Xp. For instance, Y might be a quality measurement and the X's might be peak areas or heights. Alternatively, following Mosleth and Uhlen [187], the X-variables might simply be absorbances or densities over a grid of p retention times. Often p is larger than the sample size n, so some kind of variable selection or combination is needed to fit a regression model.

PLS regression first selects a unit vector u such that the projections of the vectors of X-variables on u have maximal covariance with the Y-values [188]. The projections of the X-vectors on u are called X-scores, and they are used as regression variables in place of the raw X data. One can iterate the process by using residuals from the regression of Y-values on the scores and residuals from projections of the X-vectors on u to select further basis vectors and scores orthogonal to those previously selected. It is usually suggested to use a cross-validation method [189] to determine the number of basis vectors ($< n$) to select.

Mosleth and Uhlen [187] reported on the use of PLS regression to predict Zeleny sedimentation from the electrophoresis pattern. A novel feature of this study was the direct use of the discretized densitometry trace in the calibration, made possible by the way in which PLS handles high dimensional data. They eliminated the need for peak demarcation except for identification of three reference peaks for alignment purposes.

PLS has proven most useful in contexts where empirical prediction equations are desired, and there is little interest in isolating the effects of different variables in the regression. A typical example is determination of the concentration of an analyte based on a spectrum with relatively broad peaks [190]. The usual confidence intervals from linear regression theory do not apply to PLS regression, because the constructed regression variables (the X-scores) depend also on Y. The situation is similar to that in stepwise regression, where significance levels of t-tests and the coefficient of determination are inflated due to selection of variables in the regression. In certain cases cross-validation or other resampling methods such as the bootstrap [191] provide a means for computing significance levels and variance estimates. Martens and Naes [180] provided an overview of PLS and other multivariate calibration methods and many further references.

Although there have been many reports of success using PLS, it is important to be aware of limitations of the method. There is a tendency to treat X- and Y-scores as surrogate data, plotting them on scatter plots, reporting coefficients of determination or percentages of explained variation from the regression, and so on. However, X- and Y-scores have very different properties from the usual data in regression due to their dependence on both X and Y. In particular, the slope of the PLS regression is always positive, and scatter plots and coefficients of

determination often overstate the strength of the regression relationship. An example by Simpson *et al.* [164] considered one-factor PLS regression to relate quality measurements to RP-HPLC chromatograms of 12 hard red spring wheats. Significance levels were studied for the naive *F*-test, which is in one-to-one correspondence with the coefficient of determination (*R*-squared). It was found by Monte Carlo simulation that nominal significance levels of the *F*-test were too small by a factor of four. Although correct significance levels are easily computed by simulation methods, the example demonstrates that the visual impression created by scatter plots of PLS scores can be misleading.

Another limitation of the method relates to the way in which the PLS covariance criterion combines variation with correlation (covariance = variance × correlation). This makes it difficult to interpret relative weights of different variables in the PLS basis vectors: weight may be large due to large variance and modest correlation, or strong correlation and modest variation. Further discussion and examples were given in ref. 164. If we wish identify strong quality peaks, simpler procedures such as stepwise regression or principal component regression with variable selection may be more effective.

4.7. Confounding and the value of follow-up studies

Wheat varieties are often selected for study based on availability. Thus conclusions developed from a particular dataset may not generalize to the population of varieties that could potentially be produced. Often the best one can hope for is operational success: if certain peaks are flagged as important in a study, one relies on follow-up studies to determine whether the effect persists in other varieties. If the goal is to produce wheat with desired properties, then one uses the study results to indicate directions for breeding experiments to improve the quality of wheat. If results confirm expectations, operational success has been achieved. If not, that is also valuable information pointing to possible selection bias or confounded effects in the original study.

The complexity of wheat proteins poses an added difficulty in the interpretation because the number of peaks is usually larger than the number of varieties in the study. To illustrate, suppose we have 20 varieties and for each we record heights of 100 peaks. The 100-dimensional vectors of peak heights for the different varieties necessarily lie in a 20-dimensional space of vectors. From the data at hand we can identify no more than twenty distinct combinations of peaks to "explain" baking qualities of the wheats regardless of whether we use multiple linear regression with variable selection, or a latent effects approach such as principal component regression or PLS regression. In the simplest case two peaks may be nearly perfectly correlated in the sample solely because of varieties selected. There is no way to isolate effects of these two peaks without more data to break the correlation. More generally there will always be linear combinations of peak heights that are perfectly correlated with each other if the number of peaks exceeds the number of varieties.

In the statistical design of experiments this kind of equivalence between variables is known as confounding [192]. When confounding is present one is forced to make modeling assumptions to make further progress. A typical assumption is that certain variables are null, *e.g.*, that higher order interactions are absent or negligible in a factorial experiment. The variable selection approach in multiple linear regression of wheat quality on peaks assumes that only few peaks are associated with wheat quality, whereas the PLS approach assumes a small number of combinations of peaks bear a relationship with wheat quality. In either case, however, selection of variables is dependent on the data at hand, and follow-up studies are necessary to determine if indicated peaks are indeed important.

5. CONCLUSIONS AND PERSPECTIVES

The last decade has seen the development and refinement of excellent electrophoresis and chromatography techniques and the use of these methods to characterize heterogeneous wheat proteins. Separations are based on size, charge, surface hydrophobicity, or combinations of these traits. Depending on which methods are used, the analyst can balance analysis time, number of samples analyzed, and resolution. Undoubtedly further improvements in speed, sensitivity, reproducibility, and automation will occur, especially in techniques such as narrow-bore HPLC and capillary electrophoresis.

The remarkable separations of gluten proteins

now possibly indicate that data analysis is as important and challenging as the separations themselves. Data quickly and reliably captured contain a wealth of qualitative and quantitative information. Until recently, only obvious relationships were discovered and used. Now, investigators are beginning to use many innovative statistical and computer programs to interpret data, with valuable and sometimes surprising consequences. Careful examination of these methods shows their value, but emphasizes that caution and proper use is necessary to avoid anomalous results. We anticipate that further work will make proper combinations of analytical methods and data analyses more apparent, enhancing the use of wheat protein chromatography and electrophoresis for breeding, marketing, processing, and quality control.

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Review

Chromatographic and electrophoretic methods used for analysis of milk proteins

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ABSTRACT

Current knowledge of milk proteins and their behavior in dairy foods is based on early applications of chromatography and electrophoresis. Electrophoretic identification of the number and genetic variety of milk proteins inaugurated a research effort in which chromatographic techniques were successfully applied to the isolation of each milk protein, thus facilitating the characterization and further study of milk and dairy products. This review focuses on recent applications of chromatography for separations and analysis and on analytical applications of electrophoresis.

CONTENTS

1. INTRODUCTION

Milk is a basic food for humans providing essential nutritional components (proteins, fats, carbohydrates, minerals, and vitamins). Humans consume milk and milk products of many animal species: cow, zebu and yak (genus *bos*); water buffalo; goat; sheep; horse; donkey; reindeer; and camel [1]. Cows' milk (genus *bos*) is the most intensively studied. Bovine milk contains 3–3.5% (w/v) protein. Caseins are 2.4–2.8% of fluid milk; whey proteins are 0.5–0.7% [2]. Other classes [3] of protein present are MFGM protein, minor proteins, and enzymes.

Casein exists in milk as a casein micelle with an average diameter of about 140 nm [4]. The exact structure of the casein micelle and the forces responsible for its stability are still speculative and many models of the micelle have been proposed [5]. The casein micelle contains the four caseins: α_{s1}-, α_{s2}-, β- and κ-casein in the approximate ratios of 39:10:36:13 [6]. The primary sequence and phosphorylation sites of the four caseins are known, as well as some of the insertions, deletions and substitutions that comprise casein genetic variants [2]. The only glycosylated casein, κ-casein, contains various amounts and types of carbohydrates [3].

Proteolytic products of the four primary caseins are also present in milk. γ-Caseins (see ref. 2 for correct nomenclature) and some of the proteose-peptone components are β-casein fragments, created by the action of plasmin, the endogenous alkaline milk protease. λ-Caseins are probably α_{s1}-casein fragments also arising from plasmin cleavage. The glycomacropeptide and *para*-κ-casein are κ-casein fragments created by the action of chyomsin (rennet) [2].

Whey contains proteins soluble at pH 4.6 and

20°C, including β-LG (0.2–0.4%, w/v, milk), α-LA (0.06–0.17%, w/v, milk), BSA (0.04%, w/v, milk) and immunoglobulins (IgG, IgA, IgM; 0.04–0.09%, w/v, milk) [2]. Minor proteins are also present: lactoferrin, lactoperoxidase, enzymes, protein components of the MFGM, proteose–peptone components and glycomacropeptide. The primary sequences of α-LA, β-LG and BSA are known.

The MFGM proteins are not as well characterized, although several enzymes of the MFGM surface are known to be covalently linked to phosphatidylinsoitol [7]. Keenan *et al.* [8] reported up to 37 different polypeptides occurring in MFGM, of which seven are glycosylated.

Milk proteins are usually separated into classes before chromatographic or electrophoretic analysis. The casein micelles can be prepared from skim milk by ultracentrifugation or gel permeation chromatography; whole casein may be precipitated from skim milk at pH 4.6, a treatment that disrupts the micelles leaving whey proteins in solution. Whey proteins also remain in solution after rennet coagulation. In addition to gel filtration, ultrafiltration, microfiltration, reverse osmosis, and electrodialysis, a variety of complexing and precipitation techniques can be used to isolate whey proteins. MFGM proteins are isolated from washed cream and are separated from the fat by centrifugation.

Chromatographic techniques have been widely used for the isolation of milk proteins, and high performance methodology now forms the basis for several accurate methods of analysis. Electrophoresis has played a major role in the study of milk proteins and has been an integral part of research on the genetic variants of the major protein components of milk. Indeed, the designations of the caseins are derived from electrophoretic analysis. The minor casein components, γ_1, γ_2, γ_3, and *para*-κ-casein were discovered by electrophoresis. Research on the use of faster and more accurate and versatile chromatographic and electrophoretic techniques in milk protein analysis continues as does the evolution of such techniques. Capillary electrophoresis is being evaluated for milk proteins but nothing has been reported.

2. CHROMATOGRAPHY

2.1. Ion-exchange chromatography

Table 1 lists recent references, the type of column used, and the protein class that was separated. This list includes both anion and cation; preparative and high-performance chromatography.

2.1.1. Anion-exchange chromatography

2.1.1.1. Preparative separations

2.1.1.1.1. Caseins. DEAE-Cellulose is the most commonly used anion-exchange resin for separation of individual caseins. Four classical methods are used: Thompson [9], Mercier *et al.* [10], Davies and Law [11] and Andrews and Alichanidis [12]. Three of these methods [9,10,12] use ME in both the sample solvent and eluting buffers to reduce the disulphide bonds in κ- and α_{s2}-caseins. In addition, their eluting buffer (pH 7) contains 3.3 *M* urea. The major differences are in the NaCl gradients used to elute the caseins. Fig. 1 shows a typical DEAE-cellulose chromatogram of whole casein from bulk milk.

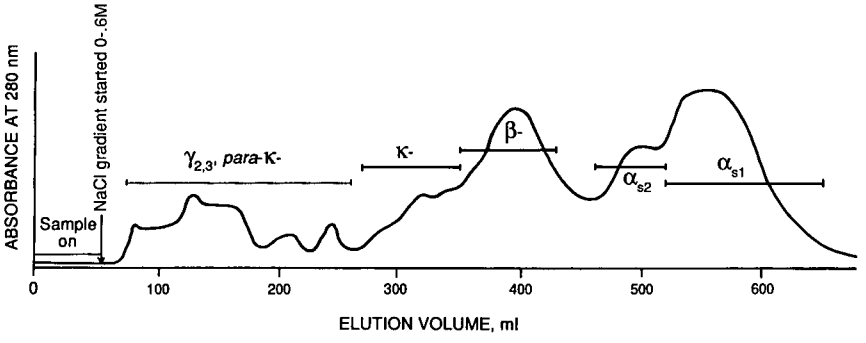

Fig. 1. DEAE-Cellulose chromatogram of whole casein. Eluent is 0.01 *M* imidazole, 3.3 *M* urea, 6 m*M* 2-mercaptoethanol pH 7, NaCl gradient from 0 to 0.35 *M*. Adapted from ref. 16.

TABLE I

SUMMARY OF ION-EXCHANGE CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
Anion-exchange chromatography —preparative separations		
QAE ZetaPrep	Casein	15
DEAE-Sepharose	Casein	13, 14, 93
DEAE-cellulose	Casein[a]	9–12, 16, 25, 93, 107, 173–175
DEAE-cellulose	Proteose–peptone	12
DEAE ZetaPrep	Casein peptides	176
QAE-Sephadex A-25	Casein peptides	177
Spherosil QMA	Whey	18
QAE ZetaPrep	Whey	23
DEAE-Selectacel	Whey	19
DEAE-cellulose	Whey	20, 21
DEAE-Toyopearl	Whey	22, 101
DEAE-Toyopearl	Minor proteins	87
DEAE-cellulose	Minor proteins[b]	178
Anion-exchange chromatography —analytical separations		
Mono Q	Casein	13–15, 25, 30, 26–28, 32, 46
Mono Q	Renneted casein	31
TSK DEAE-5PW	Casein	25, 33, 34
TSK DEAE-5PW	Casein[b]	35
Aquapore AX-300	Casein	16
Mono Q	Milk and cheese[a]	29
Mono Q	Casein peptides	177
Methyl iodine-PVIate	Casein peptides	62
Mono Q	Whey[a]	25, 26, 36–39
QPVI and KPVI[c]	Whey	40
TSK DEAE-5PW	Whey	25
Cation-exchange chromatography —preparative separations		
SP-ZetaPrep	Casein peptides	176
SP-Toyopearl	Minor proteins	44, 45
CM-Toyopearl	Minor proteins	43, 45
CM-cellulose	Whey[a,b]	42
Cation-exchange chromatography —analytical separations		
Mono S	Casein	26, 30, 46. 47
Mono S	Renneted casein	31, 48
Mono S	Whey	26
Mono S	Minor protein[a,b]	42
Mono S	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.
[b] Human milk proteins.
[c] See reference for column modification details.

Davies and Law [11] alkylated whole casein with iodoacetamide, after reduction with ME, and used an eluting buffer (pH 8.6) with 6 *M* urea.

A recent advance in casein separation methodology is the use of DEAE-Sepharose which allows higher flow-rates than does DEAE-cellulose. Barrefors *et al.* [13] used 3.3 *M* urea and ME in the buffers and obtained good separation of the caseins on DEAE-Sepharose. Christensen and Munksgaard [14] used 6.6 *M* urea and dithiothreitol in the buffers and reported good separation of the caseins except for α_{s1}- and α_{s2}-casein which could be separated on DEAE-Sepharose using 3.3 *M* urea and dithiothreitol in the buffer. Mass ion exchange has also been

used to separate caseins on a preparative level [15]. However, α_{s1}- and α_{s2}-caseins were not separated on a QAE ZetaPrep system with a pH 8, 4.5 *M* urea–ME buffer.

A single pass through a DEAE-cellulose column usually yields pure preparations of β- and α_{s1}-caseins [16]. The α_{s2}-casein fraction and the κ-casein fraction needed additional chromatography; α_{s2}-casein can be separated from contaminants, mainly β-casein and an unknown protein. The κ-casein fraction contains γ-caseins and *para*-κ-casein. Mercier *et al.* [10] separated κ-casein B, prepared by a precipitation method, into seven peaks by chromatography on DEAE-cellulose with a NaCl gradient.

2.1.1.2. Whey proteins. One technique used to isolate whey protein concentrate industrially is anion-exchange chromatography of sweet or medium acid whey, *i.e.*, whey from cheese such as Cheddar, Swiss or Mozzarella, manufactured with rennet (pH > 5.5) [17]. Proteins from the sweet whey are absorbed on Spherosil QMA, a silica-based strong anion exchanger [18]. Non-protein components, such as lactose, are eluted with water whereas the proteins require a pH < 4.5.

Anion-exchange chromatography is also used to isolate the major whey proteins and their genetic variants. β-LG A and B [19] and β-LG A and C [20] are separated using DEAE-cellulose at pH 5.8 with a NaCl gradient. α-LA and β-LG A and B were separated from dialyzed acid whey or ammonium sulphate treated whey by DEAE-cellulose chromatography [21] with a pH 7.2 Tris–HCl gradient. BSA was contaminated by an unidentified protein. Yoshida [22] used DEAE-Toyopearl columns at pH 6.8 and 8.5 and a linear NaCl gradient to separate whey fractions previously isolated from a Sephacryl S-200 column. At pH 6.8, β-LG A and β-LG B could be separated but α-LA was not retained on the anion-exchange column. At pH 8.5, α-LA separated from small quantities of β-LG and an unidentified protein. Imafidon and Ng-Kwai-Hang [23] used a QAE-ZetaPrep cartridge to separate residual α-LA from a 3% TCA supernate [24] β-LG preparation by eluting with pH 6.0 phosphate buffer and NaCl. Under these conditions α-LA did not bind to the ion exchanger; β-LG did but β-LG A did not separate from β-LG B.

2.1.1.2. Analytical separations

2.1.1.2.1. Caseins. Analytical (high-performance) liquid chromatography of caseins on strong anion-exchange columns, *i.e.*, Mono Q 50 mm × 5 mm I.D. [13,25–28], is based on techniques of DEAE liquid chromatography. Elution protocols evaluated include the use of pH 7 to 8.5 buffers that contain 3 to 8 *M* urea; NaCl gradients; and ME in the sample [25] and in both sample and buffer [13,26]; and alkylation of the casein [28]. Separation of α_{s2}-, α_{s1}- and α_{s0}-caseins presents the major difficulty. In addition to shallow salt gradients (2–5 m*M*/ml) [13,28] in the α_s region of the chromatogram, a concentration of urea just sufficient (3 to 4.5 *M*) [26] to dissociate the caseins allows for reproducible separation of these three caseins. Reported recoveries of proteins from Mono Q columns were greater than 95% [25,28]. Separation of β-casein B from other β-casein variants has been reported also [27].

Chromatography of milk on Mono Q allowed detection of cow's milk in goat's milk (1–2%) and in ewe's milk (2–4%) but goat and ewe milk could not be distinguished from each other [29]. In cheese extracts, the detection limit of cow's milk is 10% in 3-month-old Gouda type goat cheese using bovine α_{s1}-I-casein (a rennet peptide of α_{s1}-casein) as a marker [29]. Mono Q columns can be used to purify a κ-casein without the use of ME or alkylating agents [30], to identify κ-casein genetic variants and their glycosolated forms [31], to quantify individual caseins [32], and to confirm casein separation in a mass ion-exchange procedure [15].

Weak anion-exchange columns also are used to separate caseins [16,25,33,34]. For example, TSK DEAE-5PW column can separate the α_s-caseins with 3.3 and 4 *M* urea buffers and a linear NaCl gradient [33,34] as well as separate human caseins according to their degree of phosphorylation [35]. Separations on the TSK DEAE-5PW column take about twice as long as on the shorter Mono Q 5/5 column. Strange *et al.* [16] reported poor separation of α_{s1}- and α_{s2}-casein and extremely poor column stability for an Aquapore AX 300 column.

2.1.1.2.2. Whey proteins. Chromatography on the Mono Q column can be used to separate whey proteins [25,26,36–39]. Humphrey and Newsome [25] investigated buffer systems from pH 6 to 8.5 and found that pH 6 and a NaCl gradient separated α-LA, β-LG A, and β-LG B with 96% recovery from

dialyzed acid whey. The separation [36] and quantification [37] of α-LA, BSA, β-LG A and β-LG B in both sweet and acid wheys from reconstituted skim milk powder is done by using water at pH 6.6 for a buffer and eluting with a gradient to 0.7 *M* sodium acetate. Andrews *et al.* [26] separated the whey proteins, including BSA and immunoglobulins, at pH 7 with a NaCl gradient on the same type of column. Conditions were similar to those used for casein except no urea or ME was used. Addition of urea to buffers caused a loss in resolution with the whey proteins. Girardet *et al.* [38] found that, within the pH range 6 to 8, whey proteins eluted in the following order regardless of buffer system: immunoglobulins < α-LA < β-LG B < β-LG A. The resolution of BSA depends on pH and resolution of β-LG A and B depends on the steepness of the salt gradient. Laezza *et al.* [39] used a pH 7 NaCl gradient to separate and identify bovine and ovine whey proteins. Chaufer *et al.* [40] used retention of α-LA, BSA, β-LG, and lysozyme on a silica-based strong ion-exchange column to predict behavior with ultrafiltration membranes. Whey proteins also have been separated on a DEAE-5PW weak anion-exchange column with pH 7.2 NaCl gradient [41].

2.1.2. Cation-exchange chromatography

2.1.2.1. Preparative separations—whey and minor proteins

Cation-exchange liquid chromatography is used to isolate whey proteins and some of the minor proteins. Spherosil S, used in a manufacturing process [17], absorbs whey proteins from acidic whey (pH < 4.5) and then releases them when the pH is raised. Lactoferrin and lactoperoxidase can be separated from each other and acid whey proteins by chromatography on carboxymethyl cellulose at pH 7.8 [42]. Carboxymethyl-cellulose chromatography is also used to separate lactoperoxidase and lactoferrin in a whey fraction [43] and sulphopropyl cation-exchange chromatography is used to prepare lactoperoxidase from lactoferrin directly from colostral whey [44] and from sweet and acid whey [45].

2.1.2.2. Analytical separations

2.1.2.2.1. Caseins. A Mono S column, a cation exchanger, separates caseins at pH 3.5, 8 *M* urea and ME [26] with a NaCl gradient. Order of elution is: β-casein < κ-caseins and λ-caseins < α_s-caseins. α_{s2}-Casein did not separate from α_{s1}-casein. At pH 5–7, 6 *M* urea, a similar elution profile was reported [31]. Unreduced κ-casein separated from other caseins when eluted with 3.3 *M* urea, pH 3.5, octyl-glucoside (a non-denaturing detergent), and a NaCl gradient on a Mono S 10/10 column [30]. α_{s1}-Casein eluted before α_{s2}-casein at 6 *M* urea, pH 5, ME, and a very shallow NaCl gradient [46]. Genetic variants β-casein A^1, A^2, and B also could be separated [47].

2.1.2.2.2. Whey and minor proteins. High-performance cation-exchange liquid chromatography was used at low pH values to analyze the glycomacropeptide [48]. At higher pH values the same technique was used to compare human and bovine lactoperoxidase and lactoferrin as well as to confirm the separation of porcine lactoferrin from porcine colostral whey [49].

2.1.3. Recommendations

Preparative anion-exchange liquid chromatography with urea and ME is used to isolate relatively pure individual caseins in milligram to gram quantities. κ-Casein genetic variants and glycosylated moieties have also been isolated with this method. High-performance anion-exchange liquid chromatography is used for the rapid analysis of caseins. This technique separates only one of the known β-casein variants, but non-bovine caseins are readily separated from bovine caseins. However, casein peptides are poorly resolved compared to other chromatographic techniques. Both preparative and high-performance anion-exchange chromatography can separate α-LA, β-LG A and β-LG B. The simultaneous analysis of milk for whey proteins and caseins without prior separation has been unsuccessful because anion-exchange chromatography requires urea to dissociate the caseins.

Conditions for casein separation and analysis in cation exchange are not as well defined as in anion exchange. However, recent work shows that the determination of some β-casein variants is possible. Cation-exchange chromatography is useful for the separation of the minor proteins in milk.

2.2. Reversed-phase chromatography

Table 2 lists the proteins separated and columns

TABLE 2

REVERSED-PHASE ANALYTICAL (HIGH-PERFORMANCE) LIQUID CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
C_{18}	Casein	33, 50, 52
C_8	Casein[a]	13, 16, 53
C_4	Casein[a]	51, 54, 73
Phenyl	Casein	33
C_2–C_{18}	Casein peptides	179
C_{18}	κ-Casein peptides	48, 85
C_{18}	Casein peptides[a]	52, 55–59, 175
C_8	Casein peptides	60
C_4	Casein peptides	61
C_{18}	Cheese[a]	63–67, 69, 70
C_8	Cheese	67, 68
C_{18}	Whey proteins	50
C_8	Whey proteins	71
C_6	Whey proteins	72
C_4	Whey proteins[a]	51, 73–75
C_{18}	Whey peptides[a]	180, 181
C_4	Milk protein in meats	76
Ultrapore	Rennet whey in skim	77
Protein Plus	Milk powder	77
Phenyl	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.

used for reversed-phase high-performance chromatography of milk proteins.

2.2.1. *Analytical separations*

2.2.1.1. *Caseins*

Large-pore C_4, C_8 and C_{18} reversed-phase columns give excellent analytical separations of whole casein. The solvent system usually used is TFA–water–acetonitrile (0.1:70:30) with an acetonitrile gradient to 0.1:50:50. With a C_{18} column [33], all the caseins eluted in about 30 min with the order of elution being, κ-, α_{s2}-, α_{s1}- and β-casein. With a more complex acetonitrile gradient [50] and a C_{18} column, carbohydrate-free κ-casein separates into its A and B variants, α_{s1}-casein into its A, D and B/C variants (α_{s1}-casein B and C could not be separated) and β-casein into its A^1/C, A^2, A^3 and B variants. A variant designated β-casein X was also separated from bulk milk. Caseins separate in the same order on a C_8 column [16] except that α_{s1}-casein separates into two equal peaks and the β-casein variants are not as well resolved. Parris *et al.* [51] used a C_4 column, but no separation of casein variants was reported.

A C_{18} reversed-phase column, with pH 7.2 phosphate SDS buffer and propanol as eluting solvents, separates, in order of increasing retention time, α_{s1}-, β-, α_{s2}- and κ-casein [52]. One of the whole casein preparations analyzed showed two β-casein peaks with identical isoelectric focusing properties. However, one of the caseins had a leucine substituted for a proline, a mutation that would not be detected by electrophoretic analysis.

Reversed-phase columns, C_8 [53] and C_4 [54], have been used to confirm the existence of caprine α_{s1}-casein. Both of these columns gave the same order of elution, namely κ-, α_{s2}-, α_{s1}-, β-casein; the same order as found for bovine casein.

The proteins from skim milk [50] and from non-fat dry milk [51] have been analyzed without prior separation of caseins and whey proteins. On a C_{18} column [50], α-LA separated from α_{s1}-casein but α-LA had the same retention time as β-casein B; β-LG A and B were well resolved. On a C_4 column [51], α-LA appeared as a trailing shoulder on the α_{s1}-casein peak; but β-LG A and B were well resolved. In the same study, a complex of BSA, α-LA, β-LG, κ- and α_{s2}-casein, formed on heat treatment of the non-fat dry milk, eluted at the highest acetonitrile concentration of the chromatographic run. Fig. 2 shows the separation of caseins

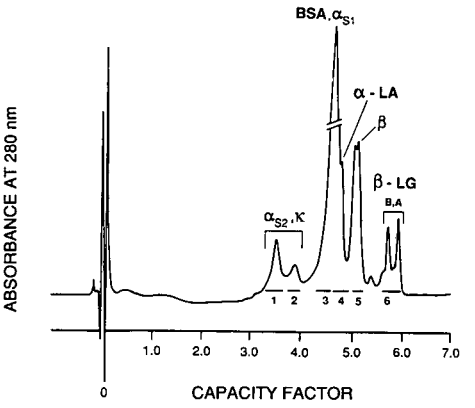

Fig. 2. C_4 Reversed-phase chromatogram of non-fat dry skim milk. Eluent is TFA–water–acetonitrile (0.1:70:30) to (0.1:40:60). Capacity factor is calculated from the position of peak maximum (t_R) and the dead time (t_0) of the column. Capacity factor equals $(t_R - t_0)/t_0$. Courtesy of N. Parris (unpublished data).

and whey proteins of a non-fat dry milk on a C_4 column.

2.2.1.2. Casein peptides

C_{18} columns in conjunction with acetonitrile gradients separate β-casein tryptic peptides at pH 2.1 [55] and pH 6.5 [56]. Tryptic and chymotryptic digests of α_{s1}-casein B are resolved with isocratic pH 2.1 95% acetonitrile [57]. Over 200 peptides were identified in casein hydrolysates [58,59] employing acetonitrile gradients at pH 2.1 and 6. C_8 chromatography [60] of a β-casein tryptic digest using pH 2.1 acetonitrile gradient resolved seven major peaks. C_4 chromatography at pH 2.1 of tryptic and chymotryptic digests of β-casein and α-LA was used to identify sites that are phosphorylated with casein kinase [61]. In comparison to size-exclusion and anion-exchange high-performance chromatography, reversed-phase C_{18} is superior in separation and resolution of peptides [62].

2.2.1.3. Cheese

The separation and identification of peptides present in cheese that contribute to its flavor, that result from aging, or that are due to type of cheese, source of cheese milk or type of rennet used in producing the cheese were studied by reversed-phase chromatography. A bitter extract of Cheddar contained 44 amino acids and/or peptides [63] whose elution order from a C_{18} column was influenced by both molecular mass and hydrophobicity. Chromatographic profiles of Gouda water extracts [64], using a C_{18} column and an acetonitrile gradient, showed three peptides, produced by lactic acid bacterial protease activity on α_{s1}-casein (f1–23), that increased with increasing cheese age. Similarly, profiles of acid precipitates of Danbo and Havarti cheeses [65] showed differences in degradation of α_{s1}- and β-casein with type of cheese, in age of cheese, source of the cheese milk and type of rennet. Water soluble and low molecular weight peptides of blue cheese [66], separated by C_{18} RP-HPLC with an acetonitrile gradient, increased as the cheese aged.

RP-HPLC was investigated as a tool for the evaluation and identification of cheeses. A C_8 column, using isocratic elution with 0.1 *M* phosphate, pH 6 was used to determine Cheddar cheese age [67]. A 65.9% correct prediction of age was achieved. A C_8 column using a ternary gradient system with an initial solvent of 0.1% TFA in water–acetonitrile–methanol (96.8:1.2:2) to a final ratio of solvents (56.3:30.3:13.4) with 0.1% TFA [68], and an internal standard of *p*-dimethylaminobenzaldehyde, could classify Cheddar, Edam, Gouda, Swiss and Parmesan correctly more than 90% of the time using multivariate analysis of HPLC data. Water extracts of Cheddar were used to evaluate the performance of four different types of C_{18} RP columns [69] and C_{18} RP-HPLC was used [70] to evaluate differences in cheese extraction procedures.

2.2.1.4. Whey proteins

Whey proteins can be separated and measured with reversed-phase columns. BSA, α-LA and β-LG were separated and identified [71] using a C_8 column and an isopropanol gradient. A C_6 column using an acetonitrile gradient [72] separated β-LG A, β-LG B, and partly, β-LG C as well as BSA and α-LA. Whey proteins from skim milk (BSA, α-LA, immunoglobulin G with minor β-casein fragment contaminants, β-LG B and β-LG A) [73], and β-LG in a model system [74], separated and were quantified on a C_4 column with an acetonitrile gradient and the amount of whey denaturation due to heat treatment determined. A short (5 cm) C_4 column was used to detect the presence of a bovine α-LA peak [75] in a 10% cow–90% ewe milk cheese. RP-HPLC can be used to detect the presence of casein and whey proteins in unheated meats [76] and rennet whey in skim and buttermilk powder [77].

2.2.2. Recommendations

Reversed-phase C_4, C_8 and C_{18} wide-pore (300 Å) silica-based columns separate the caseins, whey proteins and their various digests. Generally, resolution improves as hydrocarbon chain length of the column increases and quantitation is acceptable. Column chromatographic analysis of low-molecular-mass cheese peptides complements electrophoresis.

Caseins and whey proteins are soluble at pH < 3, especially in the presence of acetonitrile; therefore, neutral or alkaline conditions are not needed for solubility but the presence of urea and ME in the sample buffer when analyzing caseins improves resolution. Separation of milk proteins on reversed-phase columns is based on hydrophobicity of the proteins, ion exchange with the silica, as well as molecular mass effects.

Reversed-phase solvent systems are much simpler to use than those used in ion exchange. The urea used in ion-exchange buffers must be purified by passing through an ion exchanger to remove impurities before use and special equipment is needed for use with NaCl. Subsequent electrophoretic analysis from RP-HPLC is easier because non-volatile salts are absent.

2.3. Size-exclusion chromatography

Table 3 lists the recent contributions to the literature on the use of size-exclusion chromatography in the analysis of milk proteins.

2.3.1. Preparative separations

2.3.1.1. Casein micelles

Controlled pore glass beads (CPG-10 50 nm) were used to separate casein micelles from other milk proteins [78] and to separate them according to micelle size (CPG-10 300 nm) [78–82]. Before chromatography, milk samples are usually fixed with gluteraldehyde and the columns pretreated with 1% polyethylene glycol [79–82]. Buffers used for elution are Jenness–Koops [83] buffer pH 6.6 [79,80]; 5 m*M* calcium chloride, 50 m*M* NaCl, 20 m*M* imidazole pH 7 [81]; or, a synthetic milk serum containing 0.04% polyethylene glycol [78]. Fractionation of

TABLE 3

SUMMARY OF SIZE-EXCLUSION CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
Preparative separations		
Controlled Pore Glass	Casein micelle	78–81, 182
Fractosil S-1000	Casein micelle	13, 84
Sephacryl S-200	Casein[a]	174
Sephadex G-25	Casein peptides	62
Sephadex G-50	Glycomacropeptide	85
Sephadex G-75	Proteose–peptone	12
Sephacryl S-200	Proteose–peptone	102
Sephadex G-10	Cheese	86
Sephadex G-50	Cheese	63
Sephacryl S-200	Whey proteins	22
Sephacryl S-200	Minor proteins	87, 88
Sephacryl S-300	Immunoglobulins	89
Fractogel TSK HW	Immunoglobulins	89
Sephacryl S-200	MFGM proteins	91
Sephadex G-200	MFGM proteins	90
Sephadex G-10	Minor proteins[b]	178
Analytical separations		
TSK 4000 SW	Casein micelles	34, 183
TSK 4000 SW	Artificial micelles[b]	35
dual TSK 2000 SW	Glycomacropeptide	93, 184
TSK 2000 SW	Casein peptides	59
dual TSK 2000 SW and TSK 3000 SW	Whey proteins[b]	99
TSK 3000 SW	Whey proteins	95, 96
Superose 12	Whey proteins[a]	26, 37, 100, 185
GPC-100	Whey proteins	71
TSK 4000 SW	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.
[b] Human milk proteins.

casein micelles on large-pore silica gel is less successful than with CPG [81]. Sephacryl S-1000 with Jenness–Koops buffer is used to separate casein micelles [13,84].

2.3.1.2. Casein peptides

Andrews and Alichanidis [12] separated the proteose–peptone fraction into PP3, PP5 and PP8f on Sephadex G-75 and Zevaco and Ribadeau-Dumas [85] used Sephadex G-50 fine to purify the glycomacropeptides already separated by DEAE-cellulose. Lemieux and Amiot [58,59,62] used size-exclusion chromatography on Sephadex G-25 as the first step in the fractionation and identification of peptides in the hydrolysates of whole casein.

2.3.1.3. Cheese

Chromatography on Sephadex G-50 gave four fractions from an extract of bitter Cheddar cheese large enough for sensory analysis [63] and Sephadex G-10 chromatography isolated the peptides from the amino acids in a phosphotungstic acid soluble fraction of blue cheese [86].

2.3.1.4. Whey and minor proteins

Yoshida [22] used Sephacryl S-200 chromatography to isolate β-LG and α-LA from acid whey using strong acid conditions or high salt concentrations. Sephacryl S-200 chromatography was also used for isolation of xanthine oxidase and lactoferrin [87] and lactoperoxidase [88] from acid whey. Immunoglobulins and lactoferrin were isolated from colostral, cheese and acid wheys by size-exclusion chromatography on Sephacryl S-300 and on Fractogel TSK HW-55. Gel permeation on Sephacryl S-300 yielded immunoglobulin fractions of 99% purity from colostral whey, 83% purity from acid whey, and 92% purity from cheese whey [89].

Sephadex G-200 was used to separate the water soluble portion of the MFGM proteins [90] and to evaluate the efficiency of washing procedures used in the preparation of MFGM proteins [91].

2.3.2. Analytical separations

2.3.2.1. Casein micelles

Analytical (high-performance) liquid chromatography with a TSK GEL G4000SW column separated casein micelles, partially disaggregated by 6 *M* urea Jenness–Koops buffer but held in micelle form by colloidal calcium phosphate, from other milk proteins [34,35].

2.3.2.2. Casein peptides

Mottar *et al.* [92] used size exclusion on a TSK 2000 SW column (exclusion limit approximately M_r 100 000) to separate TCA-soluble peptides to identify milk suitable for UHT processing. Vreeman *et al.* [93] used two TSK 2000 SW columns in tandem to quantitate the glycomacropeptide. The TSK 2000 SW columns were conditioned with glycomacropeptide to avoid unwanted absorption of the glycomacropeptide during analytical runs. TSK 2000 SW columns were also used to separate casein polypeptides [58].

2.3.2.3. Whey and minor proteins

Whey proteins have been separated using the following size-exclusion columns: a 30 cm TSK 3000 SW (exclusion limit M_r 350 000) in tandem with a 30 cm TSK 2000 SW [94]; a 30 cm TSK 3000 SW [95]; and a 60 cm TSK 3000 SW [96]. Denaturation of whey proteins in commercial whey protein isolates and concentrates [97,98] was determined by size-exclusion chromatography on a 30 cm TSK 3000 SW column. Human whey proteins (immunoglobulins, lactoferrin, BSA, α-LA and lysozyme) were separated with dual 50 cm TSK 3000 SW and 50 cm TSK 2000 SW columns [99]. Superose 12 columns, with a reported separation range of M_r 1000–300 000 have been used to separate bovine immunoglobulins, BSA, β-LG and α-LA [26]. A comparison of caprine and bovine whey proteins by Superose 12 showed similarities between bovine β-LG and caprine β-LG and bovine α-LA and caprine α-LA. An unidentified lower-molecular-mass protein in caprine whey was the major difference found [100].

2.3.3. Recommendations

Preparative size-exclusion chromatography on CPG or Sephacryl S-1000 is the only effective way to separate casein micelles by size. One of the major difficulties with this technique is detection and evaluation of the micelle size. Size exclusion does not separate the caseins because their molecular masses are similar, but is used in preliminary separation of casein hydrolysates or of cheese extracts before

peptide mapping. Size exclusion is also valuable in the isolation of minor proteins from whey.

Analytical (high-performance) size-exclusion chromatography is useful for the separation of casein submicelles and whey proteins. However, it does not separate genetic variants of β-LG and the similarity in size between species of whey proteins probably will not permit species identification by this approach.

2.4. Hydrophobic chromatography

Table 4 lists the less commonly used types of chromatography, the specific supports and the proteins they are used for.

2.4.1. Preparative separations —minor proteins

Preparative hydrophobic chromatography is usually done to purify the minor proteins present in acid or cheese whey. Butyl Toyopearl 650M was used to purify minor proteins from acid whey [43,87,88,101]. Hydrophobic chromatography on Phenyl-Sepharose CL 4 B helped characterize the proteose–peptone fraction of milk by strongly absorbing the PP3 fraction confirming its possible origin in the MFGM [102]. Hydrophobic chromatography may be useful for separation of MFGM proteins. Lindahl and Vogel [103] exploited the hydrophobicity of calcium free α-LA to prepare α-LA from cow, human, goat, sheep and horse milks by hydrophobic chromatography. The separation was rapid and efficient. Sephadex LH-20 was used to separate bitter peptides from extracts of Gouda cheese by isocratic elution with 70% aqueous *n*-propanol [104].

TABLE 4

SUMMARY OF HYDROPHOBIC AND AFFINITY CHROMATOGRAPHY METHODS USED FOR MILK PROTEINS

Column	Protein	Ref.
Hydrophobic chromatography —preparative separations		
Phenyl Sepharose CL-4B	Proteose–peptone	102
Sephadex LH	Cheese	104
Phenyl Sepharose	α-Lactalbumin[a,b]	103
Butyl Toyopearl 650M	Minor proteins	43, 87, 88, 101
Hydrophobic chromatography —analytical separations		
Phenyl 5 PW	α_{s1}-Casein	106
Phenyl-Superose	Casein and whey	105
Affinity chromatography —preparative separations		
Thio-Sepharose 4B	Casein	108
Hydoxyapatite	Proteose–peptone	102
Sephadex G-25-Cu	Casein peptides	62
Cu^{2+}-Chelating Sepharose	Immunoglobulins	110
DNA-Agarose	Lactoferrin[a]	49
Cibacron Blue F3GA	Lactoferrin	44
Concanavalin A-agarose	MFGM protein	186
Affinity chromatography —analytical separations		
Hydroxyapatite	Casein	33
TSK chelate-5 PW/Cu^{2+}	Lactoferrin[a]	49

[a] Includes buffalo, caprine, equine, ovine, porcine and/or rat.
[b] Human milk proteins.

2.4.2. Analytical separations —caseins

Caseins were separated using a Phenyl-Superose column. Order of elution was β-, α_{s2}- and γ-, κ- and α_{s1}-casein [105]. Semipreparative hydrophobic chromatography on a Spherogel TSK-G Phenyl 5 PW column was used to purify α_{s1}-casein by removing traces of β- and α_{s2}-casein and degradation products [106]. Hydrophobic chromatography seems to be an attractive way to isolate caseins because they are extremely hydrophobic proteins but little research has been done.

2.5. Affinity chromatography —preparative and analytical separations

2.5.1. Caseins

Hydroxyapatite is classified as an affinity technique for caseins because their phosphate groups are assumed to interact with calcium and elution is carried out by increasing phosphate buffer concentrations. Hydroxyapatite was used to purify large amounts of bovine and buffalo κ-casein from whole casein [107]. Caseins eluted in the order of increasing number of phosphates: κ-casein (1P), β-casein (5P), and α_s-caseins. α_{s1}-Casein (8–9P) did not separate from α_{s2}-casein (11–13P). High-performance hydroxyapatite chromatography on whole caseins showed similar results except that the κ-casein A and B separated to some extent and the γ-caseins eluted in the solvent peak [33].

Ovine caseins were separated into two fractions on activated thiol Sepharose 4B after reducing the κ- and α_{s2}-caseins with dithiothreitol by binding to the support thiol groups. The bound caseins were eluted with cysteine [108].

2.5.2. Minor proteins

Lactoferrin and immunoglobulins can be isolated from acid and cheese wheys by metal chelating chromatography on a Sepharose 6B support with immobilized copper (Cu^{2+}) [109]. Activity of immunoglobulins was improved by alteration of the elution scheme [110].

Lactoferrin was purified by DNA affinity chromatography of urea-treated colostral whey [49]. Purity of the isolated lactoferrin was checked by HPLC on TSK gel chelate-5PW loaded with Cu^{2+}, phenyl reversed-phase, cation exchange on Mono-S and size-exclusion on TSK4000SW.

3. ELECTROKINETIC TECHNIQUES

Electrophoresis has been universally adopted for research on proteins since the introduction of polyacrylamide as a support [111,112]. The technique has progressed from separations based only on charge/mass (PAGE) to those based on molecular mass (SDS-PAGE) or isoelectric point (isoelectric focusing, IEF). Combinations of techniques providing 2D electrophoresis are commonplace. The recent introduction of precast ultrathin minigels and an automated system for performing electrophoresis, staining, and destaining (PhastSystem; Pharmacia-LKB, Piscataway, NJ, USA, and Uppsala, Sweden) has reduced analysis time from several days to a few hours and improved detection limit to 1 ng of protein.

3.1. Electrophoresis under non-denaturing conditions

The procedures of Groves for PAGE and urea-PAGE in alkaline or acid buffers [113] are still used with modification. In PAGE, proteins are negatively or positively charged, depending on buffer conditions, and migrate with mobilities related to their charge/mass ratio. For caseins, the addition of urea prevents aggregation and, at acid pH, also prevents precipitation. Urea is not considered a denaturant because the tertiary structures of caseins are more unordered than typical globular proteins. The advantages for milk proteins are that bands are well separated and genetic variants and differences in degree of phosphorylation can be readily detected. Information on relative mass is absent, however, and standards of pure caseins and/or whey proteins should be included on a gel. Moreover, there are no clues to the identity of unknown bands. Urea-PAGE has been adapted for use with the PhastSystem [114] (Fig. 3). Applications of PAGE and urea-PAGE are listed in Table 5.

TABLE 5

PAGE AND UREA-PAGE OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Milk proteins	Characterize proteose–peptone fraction	102
Milk proteins	Quantitate caseins, whey proteins	115, 116
Milk proteins	Quantitate caseins, whey proteins; compare with cellulose acetate	117
β-Casein	Proteolysis by plasmin yields γ-caseins	187
α-Casein	Determine chymosin cleavage sites	121
β-Casein	Determine cleavage sites of chymosin and pepsins	122
Bovine, buffalo casein	Comparison after rennet and other enzymes	188
Ovine casein	Determine heterogeneity	189
Whey proteins	Characterize effect of Ca^{2+} on mobility	190, 191
Buffalo whey	Characterize whey proteins	192
Cheddar cheese extracts	Effect of proteinase negative starter cultures on flavor	120
Mozzarella cheese extracts	Protein breakdown during refrigerated storage	193
Caprine milk	Detect bovine milk addition	194, 195
Ovine cheese extracts	Detect bovine milk addition	123
Ovine, caprine cheese extracts	Detect bovine milk addition	124

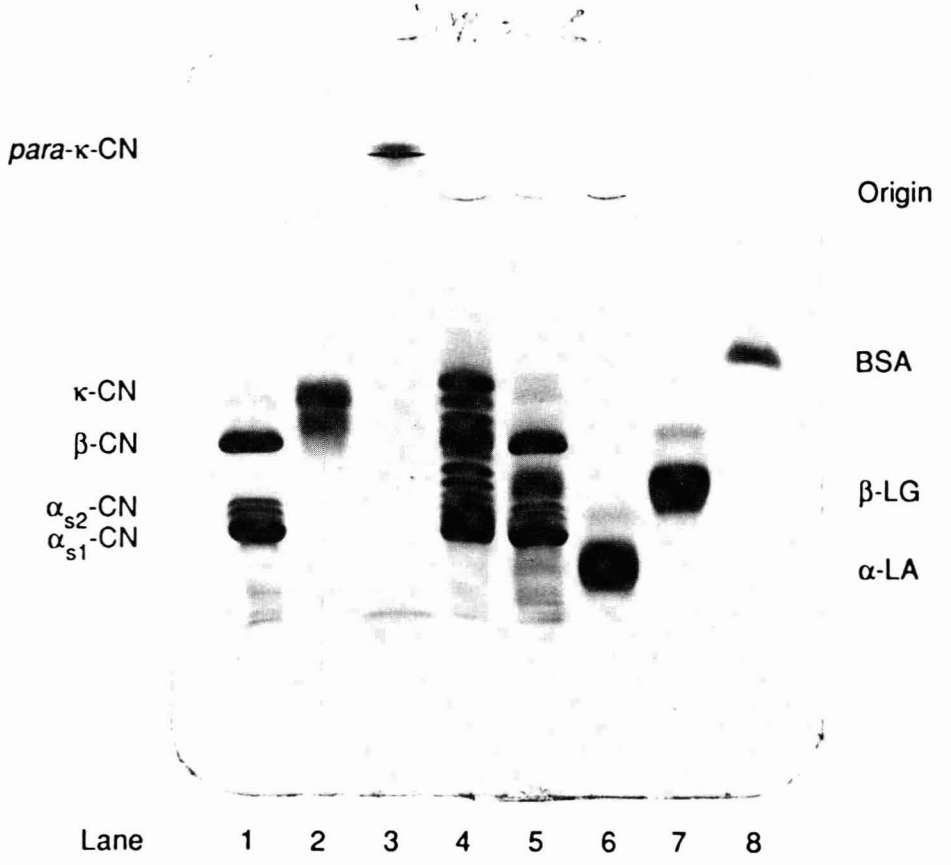

Fig. 3. Urea-PAGE analysis of bovine caseins and whey proteins on 8 to 25% gradient PhastGels and Coomassie Blue R250 staining. Samples contained 2-mercaptoethanol; buffer did not. Lanes: 1 = whole casein; 2 = κ-casein; 3 = *para*-κ-casein; 4 = 40% dephosphorylated whole casein; 5 = skim milk; 6 = α-LA; 7 = β-LG; 8 = bovine serum albumin. From ref. 114.

3.1.1. Caseins and whey proteins

After comparing polyacrylamide-agarose gels in urea-PAGE with analytical anion-exchange chromatography, Collin *et al.* [115] concluded that electrophoresis provides the greatest amount of information for quantitating bovine milk caseins, despite the 6% variability in the uptake of Coomassie Blue R250 by each casein. PAGE without urea gave better results for identifying genetic variants of β-LG [116]. Deshmukh *et al.* [117] also observed differences in absorption of aniline blue black (Amido black) among the caseins after PAGE and concluded that cellulose acetate electrophoresis was better for quantification of milk proteins.

3.1.2. Cheese

Fox [118] has reviewed many applications of electrophoresis to the analysis of cheese. Grappin *et al.* [119] reviewed studies using PAGE to analyze casein breakdown arising from the action of plasmin, starter culture proteinases and rennet. Detailed diagrams showing relative mobilities of the major peptide products are an excellent information source. In studying flavor development in Cheddar cheeses made with proteinase-negative or proteinase-positive starter cultures, urea-PAGE analysis showed that there was no correlation between flavor and casein breakdown [120]. Model studies of α_{s1}- and β-casein cleavages by chymosin [121,122] have been

important contributions to current knowledge of ripening mechanisms.

3.1.3. Detecting adulteration

The addition of bovine milk to cheese milk used for cheeses made from ewe milk [123] and from ewe and goat milks [124] was quantitated by PAGE. In both instances, the presence of bovine β-LG A and B at specific distances from the origin provided the necessary proof of adulteration. Both PAGE and IEF were equally sensitive for detecting adulteration of ewe cheese by addition of bovine and/or goat milk [125].

3.2. Electrophoresis in the presence of sodium dodecyl sulphate

The discovery that SDS binds to proteins at a ratio of 1.4 g per gram of protein [126] initiated a new class of electrophoretic separations. When disulphide bonds are broken by ME, SDS binding alters the protein chain completely. Although several structures have been proposed for SDS-protein complexes, the empirical result is that migration of the complexes in an electric field depends on mass alone. The well-known plot of log molecular mass *vs.* mobility permits estimation of protein molecular masses more easily than analytical chromatography or analytical centrifugation [127,128]. Table 6 lists specific applications.

3.2.1. Caseins and whey proteins

An SDS-PAGE method for quantitating caseins and whey proteins in processed milk powders or fluid milk, developed by Basch *et al.* [129], is based on the Laemmli procedure [130]; thiourea and 30% hydrogen peroxide were substituted for TEMED and ammonium persulphate, respectively (Fig. 4). Meisel and Carstens [131] concluded that SDS-PAGE is highly appropriate as a screening method

TABLE 6

SDS-PAGE OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Non-fat dry milk, whey protein concentrate	Quantitate caseins and whey proteins in processed milk	129
Caprine milk	Characterize proteose–peptone fraction	137
Porcine milk, colostrum	Characterize casein and whey proteins	196
Whole casein	Detect proteolytic action of *Streptococcus lactis* strains	197
α-, β-Casein	Anomalous mobility of α-casein	132
α-Casein	Plasmin cleavage sites	134
α-, κ-Caseins	Effect of plasmin	133
β-Casein	Identity of plasmin peptides with proteose–peptone fraction	12, 135, 136
κ-Casein	Association behavior	198
α-LA	Characterize effect of Ca^{2+} on electrophoretic behavior	191
Lactoferrins	Compare bovine, ovine and caprine	199
Cheese extracts	Proteolysis of Parmesan, Cheddar, Port Salut and Brie	139
Cheddar, stirred-curd cheese extracts	Effect of frozen storage	143
Low-fat Cheddar cheese extracts	Compare effects of starter culture additions	141
Cheddar cheese extracts	Effect of pasteurization on protein breakdown	140
Mozzarella cheese extracts	Compare breakdown in low-fat and high-fat cheeses	142
Dairy products	Quantitate casein and whey proteins	131
Buttermilk powder	Detect adulteration by NFDM	144
Meat products	Detect casein, whey proteins	200

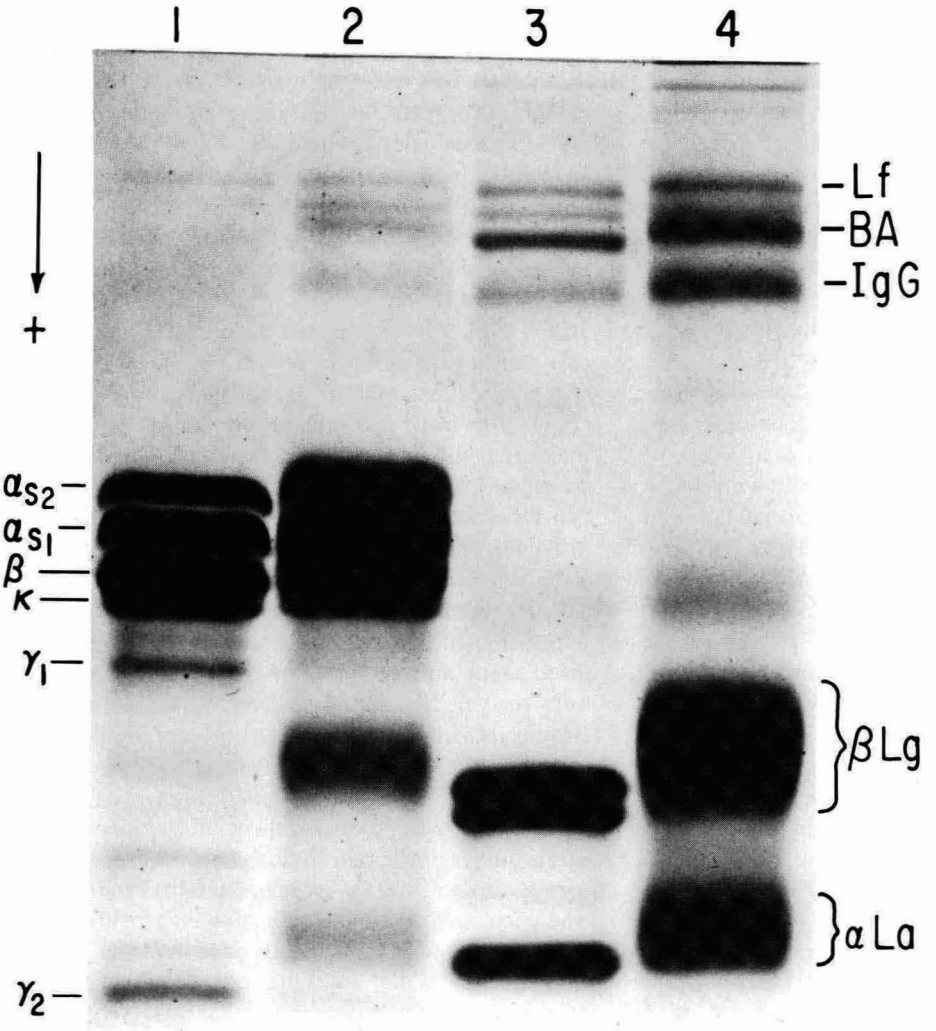

Fig. 4. SDS-PAGE comparison of laboratory-prepared whole caseins (lane 1) and whey (lane 3) with processed dairy products (non-fat dry milk, lane 2, and whey protein concentrate, lane 4). Gels were 18 × 14 cm and 3 mm thick and stained with Coomassie Blue R250. Lf = Lactoferrin; BA = bovine serum albumin; IgG, heavy chain immunoglobulin G; β-Lg = β-lactoglobulin; α-La = α-lactalbumin; α_{S2}, α_{S1}, β, κ, γ_1 and γ_2 = various caseins. From ref. 204.

for determining whey protein and casein contents of milk products.

Although proteins separate in SDS-PAGE according to mass, a somewhat anomalous behavior has been observed in the retardation of α_{s1}-casein relative to the slightly larger β-casein. Creamer and Richardson [132] found that although both α_{s1}- and β-caseins bound 1.3 g of SDS per gram of protein, α_{s1}-casein had an unexpectedly large hydrodynamic size. SDS-PAGE of pure α_{s1}-casein treated with plasmin indicated the formation of three lower-molecular-mass bands ranging from 20 500 to 10 300 [133]. Similarly, sites of plasmin attack on α_{s2}-casein were studied by Visser *et al.* [134] in the isolated protein.

The proteose–peptone fraction has been extensively investigated with SDS-PAGE [12,102,135,136]. Nearly all of this heterogenous mixture in bovine

milk can now be accounted for by plasmin-mediated proteolysis of β-casein, although Paquet *et al.* [102] have suggested that a higher-molecular-mass portion may be a fragment of MFGM. Molecular masses of the proteose–peptone components of goat milk, determined by SDS-PAGE, were similar to those of bovine [137]. This has been confirmed and extended to ewe milk, as well [138].

3.2.2. Cheese

SDS-PAGE provides valuable information for ripening studies when coupled with densitometric analysis of decreasing band intensity with time. Although casein breakdown can also be measured with PAGE, the increasing number and intensity of lower-molecular-mass bands observed with SDS-PAGE during ripening provides additional data and suggests further research. Characteristic patterns were found in SDS-PAGE comparisons of Parmesan, Cheddar, Port Salut and Brie cheeses [139]. Lau *et al.* [140] used SDS-PAGE to show that pasteurization can induce whey-casein interactions that could inhibit access of proteolytic enzymes in Cheddar cheese during ripening, thus influencing flavor development. The potential of *Micrococcus freudenreichii* or *Pediococcus pentosaceus* to accelerate ripening of low-fat Cheddar cheese at different temperatures was tested with SDS-PAGE after 6 months of ripening [141]. Tunick *et al.* [142] used SDS-PAGE to monitor breakdown of caseins and peptide formation in low-fat Mozzarella cheese during 6 weeks of storage. Basch *et al.* [143] also used SDS-PAGE to evaluate long-term frozen storage of traditional and stirred-curd Cheddar cheeses in terms of quality for process cheese manufacture.

3.2.3. Detecting adulteration

The availability of molecular mass data, in addition to separation of components, makes SDS-PAGE a method of choice for monitoring activities, especially when adulteration is suspected. For detecting adulteration of buttermilk powder, Holsinger *et al.* [144] developed an SDS-PAGE procedure based on the presence of three specific MFGM bands characterized earlier by Basch *et al.* [91]. The method can be used with the PhastSystem.

3.3. Isoelectric focusing

3.3.1. Caseins and whey proteins

Separating proteins according to their isoelectric points (p*I*) is particularly appropriate for analyzing caseins, as there are many genetic variations among and within species. Analysis of bovine β-casein genetic variants is simplified by use of IEF [145]; otherwise, both alkaline and acid PAGE would be needed to differentiate A variants from B, C and D and A^1, A^2 and A^3 from each other. Rapid methods for identifying genetic polymorphism in bovine milks were developed for the PhastSystem [146,147] (Fig. 5). In both procedures, precast IEF gels were incubated with urea and ampholytes before use. IEF on ultrathin (50 μm) polyacrylamide gels was a superior method for detecting heat-induced changes in whey proteins [148].

3.3.2. Detecting adulteration

An IEF method for determining the content of dried skim milk, casein, whey proteins, and total milk protein in compound feeds was developed by Braun *et al.* [149]. Ultrathin polyacrylamide gels containing urea were used with α_{s1}-casein B as a marker for casein and α-LA B plus β-LG A as markers for whey. Substitution of whey solids for skim milk could be readily detected. IEF for monitoring adulteration of ewe cheese with bovine milk was performed with the PhastSystem on urea-containing polyacrylamide gels cast with a special assembly [150]; 0.5% bovine milk content could be detected. The procedure has been successfully applied to detection of bovine milk in Roquefort cheese [151], even after 5 months of ripening.

IEF detection of bovine milk in ewe milk or cheese (as low as 0.5%) was enhanced by adding plasmin to generate γ_2-casein *in vitro* [152]. Monitoring *para*-κ-casein as a marker in an IEF procedure for detecting bovine milk in ewe hard cheese was successful [153], but false positive responses occurred in ripened Roquefort because of a peptide migrating with bovine *para*-κ-casein. Additional applications appear in Table 7.

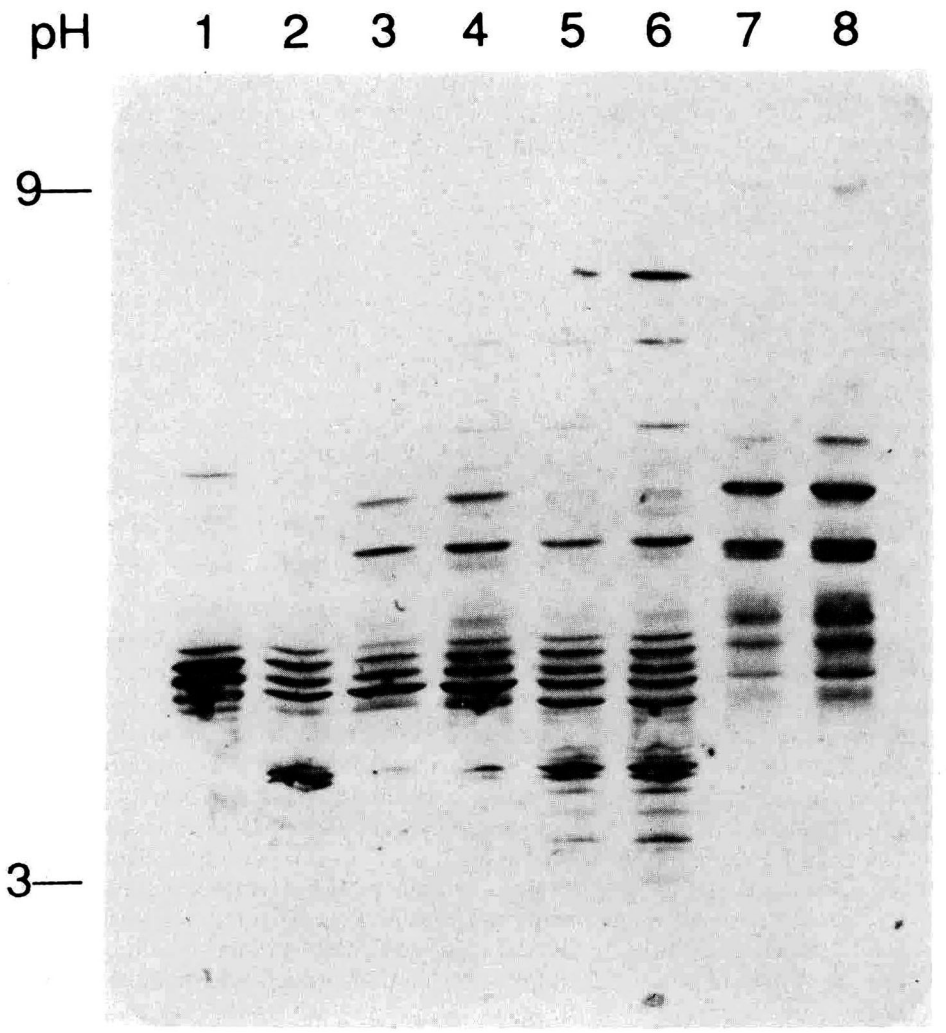

Fig. 5. IEF of bovine caseins on PhastGels using modifications described by Vegarud *et al.* [146]. The ampholyte solution (Serva) contained one part pH 4.0–6.0 and two parts pH 5.0–7.0 (v/v). Lanes: 1 = α_{S2}-casein; 2 = α-casein complex; 3 and 4 = crude β-casein; 5 and 6 = whole casein; 7 and 8 = κ-casein B. Courtesy of E. D. Wickham and H. M. Marrell, Jr. (unpublished data).

3.4. Two-dimensional electrophoresis

The combination of IEF, usually in gel rods, followed by SDS-PAGE on a slab gel (2D electrophoresis), provides the greatest differentiation among casein and whey components of milk and other dairy products. In a modified 2D electrophoresis method for whey proteins [154], IEF over a range of pH 3 to 8 was followed by PAGE without SDS in a gradient gel. However, the gradient did not compensate for the absence of denaturant, and a cluster of spots resulted. Bovine milk proteins were analyzed by IEF in the presence of a non-ionic detergent and urea with a pH range from 3 to 10; SDS-PAGE on a 14% acrylamide slab gel followed [155]. The method was used to show that high-speed centrifugation of raw skim milk results in removal of a large portion of α_S-caseins; β-casein was unchanged. The same workers used capillary tubes for IEF and a minigel for the slab to obtain enhanced

TABLE 7

IEF OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Caseins, whey proteins	Identify genetic polymorphism	146
Caseins	Identify genetic variants	145
Caseins, whey proteins	Phenotype genetic variants	147
Milk proteins	Characterize proteose–peptone fraction	102
Whey proteins	Characterize heat-induced changes	148
β-LG B, H	Characterize β-LG H	201
Lactoferrins	Compare bovine, ovine and caprine	199
Bovine, ovine milk cheeses	Characterize γ_2-, γ_3-, and *para*-κ-caseins	145, 153
Ovine cheese extracts	Detect bovine milk addition	150, 151, 202
Ovine cheese extracts	Detect bovine, caprine milk addition	125
Ovine cheese	Detect bovine milk addition by enhancing γ_2-casein content	152
Feed materials	Detect caseins and whey proteins	149

resolution in much less time [156]. To investigate the occurrence of α_{S1}-casein in goat milk, a "reverse" 2D electrophoresis procedure was used [157]. The first dimension was electrophoresis on either starch–urea or polyacrylamide–agarose. This was followed by IEF on polyacrylamide. The 2D electrophoresis procedure provided the best resolution of caseins. Additional applications appear in Table 8.

3.5. Detection of protein bands on polyacrylamide gels

Methods for staining gels seem to be more numerous and diversified than the variety of procedures for electrophoresis. Two recent reviews are recommended [158,159]. Coomassie Blue R250 has been the most widely used general stain because its sensitivity is about 10 times greater than that of aniline blue black (Amido black), it binds more uniformly to proteins, and the colors of stained bands do not change as much in stored gels [160]. Unfortunately, Coomassie blue does not obey Beer's law at high concentrations and this can lead to problems in densitometry [161]. The advent of silver stains has increased sensitivity up to 100-fold [162], but the increased ability to analyze dilute samples and observe all components of a sample can be a

TABLE 8

2D ELECTROPHORESIS OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Caseins	Effect of ultracentrifugation	155
Caseins	Effect of *Pseudomonas fluorescens*	156
Bovine, ovine and caprine milk	Characterize hydrophobic components of proteose–peptone fractions	138
Caprine caseins	Compare starch–urea, agarose–PAGE, IEF methodology	157
Caprine casein	Characterize α_{S1}- and α_{S2}-casein polymorphism	203
Ovine casein	Determine heterogeneity	189
Whey proteins	Determine p*I* values	154

mixed blessing. For regulatory applications, high levels of sensitivity are desirable, whereas protein purifications must now meet the more stringent requirement imposed by silver staining. Poehling and Neuhoff [163] have discussed the relative advantages of silver and Coomassie Blue stains.

A special technique for silver staining of PhastGels involves manual use of the Gelcode system (Pierce, Rockford, IL, USA) to give silver stained bands in several colors [164]. Similarly, in another silver stain modification [165], glycosylated proteins appear as colored bands while non-glycosylated proteins are black or grey. In addition, with double-staining techniques, Coomassie Blue R250 can be used after the silver stain (Bio-Rad, Richmond, CA, USA) to provide even greater differentiation, or a Coomassie-stained gel can be decolored and restained with silver [166]. Glycosylated proteins bind Coomassie Blue R250 very poorly but can be detected with the traditional periodic acid–Schiff stain [167], although the color intensity of the dye is low. The technique has been adapted for detection of glycoproteins on PhastGels after PAGE, SDS-PAGE or IEF [7,168,169].

The newest methodology involves the use of metal salts, such as cupric chloride and zinc chloride, which yield clear bands on a colored background [170]. The technique is especially appropriate when proteins will be recovered from gels or subjected to immunoblotting, as fixing is not required before staining and the metal salt binds reversibly [171, 172]. Copper-based reverse stains are available from Bio-Rad and BDH (Poole, UK).

4. ABBREVIATIONS

BSA	Bovine serum albumin
CPG	Controlled pore glass
CM	Carboxymethyl
2D	Two-dimensional
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
LA	Lactalbumin
LG	Lactoglobulin
ME	2-Mercaptoethanol
Ig	Immunoglobulin
MFGM	Milk fat globule membrane
M_r	Relative molecular mass
NFDM	Non-fat dry milk
P	Phosphate bound to casein
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
PP3	Proteose–peptone component 3
PP5	Proteose–peptone component 5
PP8f	Proteose–peptone component 8 fast
QAE	Diethyl-(2-hydroxypropyl)aminoethyl
RP	Reversed-phase
SDS	Sodium dodecyl sulphate
SP	Sulphopropyl
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
UHT	Ultra-high temperature

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Review

Chromatographic determination of vitamins in foods

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ABSTRACT

Chromatographic methods for the determination of water- and fat-soluble vitamins in foods are reviewed. For each vitamin, sample preparation, detection problems and chromatographic conditions are presented and discussed. High-performance liquid chromatography (HPLC) is becoming a standard method in vitamin assay, especially for routine work. HPLC systems can be automated using in-line solid-phase extraction and column switchings, resulting in very sensitive methods, even when simple UV detection is employed.

CONTENTS

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1. INTRODUCTION

A study of the literature published over the last few years suggests that chromatographic methods have become an indispensable tool for the rapid and specific determination of vitamins in foods.

Vitamins are diverse compounds with regard to structure, biological activities and their chemical properties. The water-soluble vitamin group includes thiamine and its derivatives (vitamin B_1), riboflavin (vitamin B_2), niacin (nicotinic acid) and its amide (nicotinamide, vitamin PP), vitamin B_6 vitamers (pyridoxal, pyridoxol and pyridoxamine and their derivatives), pantothenic acid, folic acid and its derivatives (folacin), biotin (vitamin H) and cyanocobalamin (vitamin B_{12}), in addition to L-ascorbic acid and L-dehydroascorbic acid (vitamin C) and other biologically active compounds, classified as pseudo-vitamins, such as orotic acid (vitamin B_{13}), 4-aminobenzoic acid, inositol (myo- and mesoinositol), rutin (vitamin P) and S-methylmethionine (vitamin U).

The fat-soluble vitamins fall into four groups of vitamin-active compounds: vitamin A (retinoids and carotenoids), vitamin D [ergocalciferol (D_2) and cholecalciferol (D_3)], vitamin E (tocopherols and tocotrienols) and vitamin K (phylloquinone and menaquinones). This means that only very rarely can they be extracted from a complex matrix such as food by a simple and rapid procedure. Hence the extraction and clean-up steps represent the rate-limitating stage in almost all vitamin analyses. Further, the extraction has to be optimized not only both for vitamin degradation evaluation and for the specific food matrix, but also by checking the recovery of added vitamins.

Undoubtedly the continuing development of HPLC, LC–MS, GC and SFC methods applied to

vitamins suggests that their positive characteristics in resolving complex analytical problems are being confirmed; there has been a continuous improvement in these techniques with respect to the optimization of both stationary phases and of hardware, including the introduction of new detectors.

In this review we present a critical assessment of chromatographic methods developed for the determination of vitamins in foods, paying particular attention to sample handling and detection problems. Even if not applied to food matrices, but to multivitamin tablets or other pharmaceutical products and standards, some new techniques such as MECC, SFC and isotachophoresis, are included in view of their potential application to foods.

For each vitamin, surveyed methods are tabulated in order to assist the evaluation for method selection.

2. SIMULTANEOUS DETERMINATIONS

The great improvement in the simultaneous determination of different vitamins is due to the application of chromatographic methods; however, the principal problem to be taken into account should be the standardization of the conditions of extraction, paying attention to the chemical/physical characteristics of the food and the stability of the vitamins to be determined. When simultaneous determinations can be carried out, the analysis time is decreased and there is a less extensive use of materials and equipment.

2.1. Fat-soluble vitamins

HPLC has been the most often applied technique over the last 5 years, even though SFC has been developed and improved.

2.1.1. High-performance liquid chromatography

Ball [1] surveyed simultaneous HPLC methods up to 1984. However, there have been futher developments with the application of microcolumn (narrow-bore) HPLC with multi-channel UV–VIS detection (Tables 1 and 2) for the determination of vitamin A, E and D and β-carotene in milk, animal feeds and vegetable oils [2,3], and of microcolumn HPLC with UV detection of vitamin A, D, E and K in margarine and butter [4]. NPC with UV and FL detectors in series was used by Kneifel *et al.* [5] to determine vitamin A and D in butter and infant formula. To eliminate the need for time-consuming and laborious solvent extraction procedures, Brown-Thomas *et al.* [6] proposed a multi-dimensional HPLC method using a 10-μm gel column, to eliminate the bulk of the lipid material, which was in-line with a semi-preparative aminocyano column for NPC. This HPLC system was arranged with a switching valve to direct the solvent flow towards one or another column. In addition, to make the determination of *dl*-α-tocopheryl acetate possible, a final off-line RP-HPLC step was included.

Singh and Bradbury [7] developed an HPLC method for the simultaneous determination of α-carotene, β-carotene, retinol and vitamin D_2 in South Pacific root crops. Hot saponification was followed by hexane extraction, then for vitamin D_2 a clean-up procedure on an open silica gel column was applied. After testing five mobile phases, methanol–ACN–$CHCl_3$ (40:40:20) showed the best resolution in RP-HPLC, achieving the separation in the order retinol, vitamin D_2, α-carotene and β-carotene within 7–8 min. UV detection was performed with a dual-wavelength detector. Average recoveries were 87%, 91%, 93% and 94% for vitamin D_2, β-carotene, retinol and α-carotene, respectively.

The replacement of liquid–liquid partitioning with liquid–solid extraction of retinol and α-tocopherol from fatty samples of feedstuffs was studied by Bourgeois and Ciba [8]. By replacing methanol with methanol–ethanol–*n*-butanol in the saponifying solution, the two vitamins were de-esterified and extracted together from the same sample by using a disposable Kieselguhr cartridge. This procedure is faster than conventional liquid repartition methods, but it gives the same quantitative recovery (100.3% retinol and 98.5% α-tocopherol).

Kim and Kim [9] evaluated alkaline and enzymatic hydrolyses and both liquid–liquid and liquid–solid extraction for the HPLC determination of vitamin A, D, E and K in foods. No difference was found between alkaline and enzymatic hydrolysis for vitamin A, D and E, but enzymatic hydrolysis gave better results for vitamin K. Diethyl ether, pentane and hexane gave the best recovery for liquid–liquid extraction and a silica cartridge was used for liquid–solid extraction. The HPLC analysis was performed on a C_{18} column with methanol–

TABLE 1

SAMPLE PREPARATION FOR THE SIMULTANEOUS HPLC DETERMINATION OF FAT-SOLUBLE VITAMINS

For analytical HPLC conditions see Table 2.

Extraction	Chromatographic clean-up		Food	Analyte	Ref
	Column	Mobile phase			
Dilution with *n*-hexane, in-line clean-up: (1) GPC to discharge lipids; (2) switching valve to HPLC column A; (3) fraction collected from (1), evaporate to dryness, dissolve in mobile phase, RP analysis with column B	GPC on PVB–PDVB polymer, 50 Å, 1 μm	30% methyl *tert.*-butyl ether–CH_2Cl_2 (30:70) in hexane	Fortified coconut oils	Vit. D_2, retinyl acetate	6
Saponify in ethanol–KOH (80–85°C, 1 h), extract with hexane, for vit. D_2 clean-up on open column, 2nd fraction collected, evaporate to dryness, dilute in hexane, RP analysis	Open column, silica gel 60 (300 × 15 mm I.D.)	75 ml benzene, 250 ml ethyl acetate–benzene (1:99), collect last 200 ml	South pacific root crops	Retinol, α-carotene, β-carotene, vit. D_2	7
KOH hot saponification in methanol–ethanol–*n*-butanol (4:3:1). Solid-phase extraction	Open column, Extralut 7, Kieselghur	Isooctane	Fatty samples, feedstuffs	Retinol, α-TOC	12

TABLE 2

ANALYTICAL CONDITIONS FOR SIMULTANEOUS HPLC DETERMINATION OF FAT-SOLUBLE VITAMINS

For sample preparation see Table 1.

Stationary phase	Mobile phase	Detection	Analyte	Food	Ref.
On-line with GPC: semi-preparative (A) NH_2-CN phase	Linear gradient: 0 min, 30% methyl *tert.*-butyl ether–CH_2Cl_2 (30:70) in hexane; 15 min, 100% methyl *tert.*-butyl ether–CH_2Cl_2 (30:70); flow, 2 ml/min	UV, 292 nm	Vit. D_2, retinyl acetate	Fortified coconut oils	6
Off-line: (B) Polymeric RP-C_{18}	Methanol–propanol–water (A) 60:10:30 (B) 89.5:10:0.5 Gradient: 0 min, 50% A; 18 min, 95% B; flow, 1.5 ml/min	UV, 284 nm	*dl*-α-Tocopheryl acetate		
5 μm C_{18} RCSS Guard-Pak C_{18}	ACN–methanol–$CHCl_3$ (40:40:20)	UV, 325 nm 280 nm 452 nm Dual wavelength	Retinol Vit. D_2 α-Carotene β-Carotene	South Pacific root crop	7
(1) 5 μm Spheri-5-RP18 guard column Aquapore ODS 7 μm	ACN–CH_2Cl_2–methanol (70:20:10), 1.8 ml/min	Programmable UV–VIS:	(a) Carotenoids (b) Retinol,	Vegetables	12
(2) 5 μm Spheri-5-ODS	ACN–methanol. Flow gradient: 0 min, 1.8 ml/min, 5 min, 3.5 ml/min	(a) 450 nm (b) 313 nm (c) 280 nm	Retinyl acetate (c) α-TOC, α-TOC acetate		

water (95:5) as eluent and detection at the highest absorption wavelength of the vitamins with a variable-wavelength UV detector.

Staroverov *et al.* [10] did not presaponify vegetable oils fortified with vitamin A, E and K_3; they separated the three vitamins on microcolumns filled with Silasorb using 6% diethyl ether in hexane as eluent and detection at 320 nm for vitamin A and K_3 and 220 nm for vitamin E.

Micali *et al.* [11] reported a procedure for the determination of α-, β-, γ- and δ-tocopherols, α-, β- and γ-tocotrienols, β-carotene, all-*trans*-retinol and retinyl palmitate in the unsaponifiable fraction of butter and margarine involving HPLC on an HS-Silica column with hexane–isopropanol (99.8:0.2) as eluent and programmable FLD ($\lambda_{ex.}$290–360 nm $\lambda_{em.}$ 330–480 nm). Olmedilla *et al.* [12] developed two NARP methods for the rapid separation of ten carotenoids, three retinoids, α-tocopherol and α-tocopheryl acetate in vegetable samples using a single sample preparation and RP-HPLC separation on a C_{18} column with two-channel UV detection. Two eluent systems were tried and discussed for their efficiency to separate standards of the carotenoid, retinoid and tocopherol classes. ACN–methanol (85:15) as eluent at two flow-rates gave the best resolution among carotenes and retinyl palmitate in tomato extracts. The DL was found to be 1 ng for β-carotene, 0.3 ng for retinol and 15 ng for α-tocopherol.

The retention behaviour of fat-soluble vitamins on silica gel was investigated by Hara *et al.* [12] with binary solvents each containing ethyl acetate, THF or 2-propanol in hexane. A linear relationship be-

tween the log (capacity factor) and log (concentration of polar solvents) was confirmed. The retention sequence of the vitamins was retinol > ergocalciferol = cholecalciferol > δ- > γ- > β- > α- tocopherol > menadione > phylloquinone. They explained this retention sequence on the basis of hydrogen bonding interactions between the active functional group on the solute molecule and the silanol groups on the silica gel surface. The 2-propanol binary systems better resolved fat-soluble vitamins using a silica gel column.

A similar study was carried out by Ando *et al.* [14] on the retention behaviour of ten fat-soluble vitamins on aminopropyl- and cyanopropyl-bonded silica columns using binary solvents containing or ethyl acetate, THF or 2-propanol in hexane. A linear relationship between log(capacity factor) and log(solvent composition) was ascertained. The retentivity of the amino-bonded phase was stronger and that of the cyano-bonded phase was weaker than that of the silica phase when hexane–ethyl acetate or THF binary systems were employed. However, silica gel and 2-propanol binary solvents generally gave a superior peak shape for all vitamin samples.

2.1.2. Supercritical fluid chromatography

White *et al.* [15] reported a rarely used application of SFC for the separation of a group of fat-soluble vitamins in foods. Capillary SFC–FID was performed with an SFC system capable of linear pressure programming of the mobile phase while operating with either capillary or packed columns, with FID at 385°C. Two columns were evaluated: DB-5 (10 m × 50 μm I.D.) fused silica at 140°C with a CO_2 mobile phase held at 150 atm for 10 min then a linear pressure programme to 200 atm at 5 atm/min and held at 200 atm; and DB-WAX (10 m × 100 μm I.D.) at 150°C with CO_2 held at 175 atm for 25 min then a linear pressure programme to 350 atm at 4.17 atm/min and held at 350 atm. The first column resolved vitamin K_3, A, E and K_1 and provitamin D within 40 min and the second resolved vitamin K_3, vitamin A acetate, vitamin E acetate and vitamin K_1, A, E and D_3 within 48 min.

2.2. Water-soluble vitamins

The simultaneous separation and determination of water-soluble vitamins have been developed over the last few years especially for HPLC techniques.

2.2.1. High-performance liquid chromatography

Most of the HPLC methods (Table 3) have been developed to determine from two to four vitamins together in standard solutions and/or simple food

TABLE 3

CONDITIONS FOR SIMULTANEOUS HPLC DETERMINATIONS OF WATER-SOLUBLE VITAMINS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add I.S. solution (acetanilide in mobile phase), dilute with mobile phase, filter	Nucleosil 7 C_{18}, 40°C	Acetonitrile–0.01 *M* KH_2PO_4–triethylamine (8:91.5:0.5) with 5 m*M* Na octanesulphonate (pH 2.8)	UV, 254 nm	Oral liquid tonics	16
Dilute with water	RP-MPS	0.1 *M* H_3PO_4–0.1 *M* Na_2HPO_4 buffer (pH 5) with 10 m*M* Bu_4NBr	UV, 270 nm	Enriched pasta	21
Defat with hexane, extract in 0.1 *M* HCl (75°C, 15 min) with sonication, adjust pH to 4.6, filter, clean-up on SCX disposable column	Supelcosil LC-8-DB, 35°C	5 m*M* Na hexanesulphonate with 0.1% triethylamine (pH 2.8)–methanol (85:15)	UV, 200 nm	Almonds	20
Centrifuge, dilute 1:10 with water, filter through 0.20-μm membrane	Spherisorb ODS-2	5 m*M* octylamine orthophosphate	UV, 254 nm	Milks	18

matrices, such as drinks [16–18], even though more complex food matrices have been studied [19–23]. Dong *et al.* [24] systematically evaluated the factors controlling the separation in the IP-RPC mode of seven water-soluble vitamins (ascorbic acid, niacin, niacinamide, pyridoxine, folic acid, thiamine and riboflavin (Fig. 1) while Dai *et al.* [25] optimized the operating parameters in the RP mode using a polynomial fitting least-squares method, studying the relationship between retention time and peak width and methanol concentration in the mobile phase, which is the main factor affecting the RP separation of water-soluble vitamins.

One of the critical stages of the simultaneous methods is the extraction and the chemical/physical characteristics of the food and as the stability of the vitamins to be assayed have to be taken into account. Most of the simultaneous methods have been developed for those vitamins which can be extracted in a single procedure from food, without suffering losses; for example, to determine thiamine, riboflavin and niacin together, the extraction is carried out mainly with acidic and enzymatic treatments, sometimes followed by clean-up on C_{18} disposable columns [21–23]. On the other hand, Rizzolo *et al.* [20] extracted six water-soluble vitamins (biotin, niacin, riboflavin, thiamine, pyridoxine and folic acid) from raw almonds using an extraction procedure involving heat sonication of defatted materials and removal of interfering compounds by strong cation-exchange column clean-up.

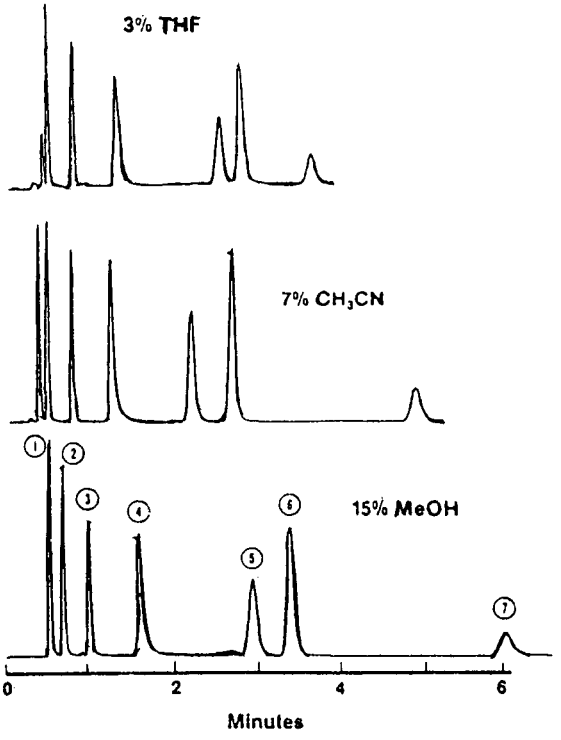

Fig. 1. Chromatograms showing the separation of water-soluble vitamins on a 3-μm end-capped spherical C_8 Pecosphere-3CR column (83 mm × 4.6 mm I.D.), using different organic modifiers + water containing 5 m*M* sodium hexansulphonate and 1% acetic acid. Peaks: 1 = ascorbic acid; 2 = niacin; 3 = niacinamide; 4 = pyridoxine; 5 = folic acid; 6 = thiamine; 7 = riboflavin [24].

Further, some published methods are not truly simultaneous, because the separation is not performed in a single chromatographic run, even though it is on the same column. For example, to determine riboflavin and thiamin in dietetic foods, Hasselmann *et al.* [22] performed two successive runs of the same extract on the same C_{18} column with an FL detector set at the proper wavelength (riboflavin $\lambda_{ex.}$ 422 nm, $\lambda_{em.}$ 522 nm and thiamine as thiochrome at $\lambda_{ex.}$ 366 nm and $\lambda_{em.}$ 435 nm).

To avoid successive runs on the same column, two or more detectors can be connected in series. To determine niacin and pyridoxine in fortified food products [26], the two vitamins were separated after acid extraction on an ODS phase and detected with UV and FLD instruments in series. To determine thiamine, riboflavin and niacin in heat-processed meats, Dawson *et al.* [23] set up an RP method in which an aliquot of the sample was directly separated on a C_{10} column, and riboflavin and niacin were detected using FLD and UV instruments in series. After derivatization of thiamine to thiochrome, the sample was separated under the same conditions and thiochrome was detected by FLD.

IP-RPC has been widely used, with different mobile phases and counter ions. However, to determine ascorbic acid and orotic acid in milks Gennaro and Abrigo [18] developed an RP ion-interaction HPLC method, using octylamine orthophosphate as the interaction reagent, without addition of an organic modifier or buffer to the mobile phase.

The RP separation on a C_{18} column of nicotinamide, pyridoxine, thiamine, riboflavin, folic acid and cyanocobalamin in oral liquid tonics, as affect-

ed by the ion-pair reagent chain length, concentration of triethylamine, pH and concentration of acetonitrile in the mobile phase, was extensively studied by Maeda *et al.* [16]. The capacity factor of thiamine was greatly influenced by the length of the alkyl chain of the ion-pair reagent, the concentration of triethylamine and pH. On the other hand, the same factors had only a slight influence on folic acid and riboflavin.

Generally, standard ODS columns are used, even though the fully end-capped columns provide a better peak shape and a better separation of the vitamins than the standard columns [17,20,26].

To shorten the time required for the simultaneous determination of ascorbic acid, nicotinic acid, thiamine, pyridoxine, hydroxycobalamin and FAD contained in beverages, Akiyama *et al.* [17] proposed three newly prepared bonded silica column packing materials [3-morpholinopropyl- (MPS)3-(1-piperazinyl)propyl- (PZS) and 3-piperidinopropyl- (PDS)silica gels], which run with one buffer solution throughout the analysis without gradient elution.

Using parallel dual-electrode ED at two different oxidative potentials, only selected vitamins could be determined [27,28]. For example, with one electrode controlled at +0.8 V (*vs.* SCE) and the other at +1.2 V (*vs.* SCE), Hou and Wang [28] successfully determined ascorbic acid, pyridoxine, pyridoxamine, *p*-aminobenzoic acid and folic acid in multivitamin tablets without interference from other water-soluble vitamins. Their HPLC–ED system offered superior selectivity and improved sensitivity, the DL being 20 pg (ascorbic acid), 40 pg (*p*-aminobenzoic acid), 0.2 ng (folic acid), 0.6 ng (pyridoxine) and 1 ng (pyridoxamine). Not all the methods are suitable for determining all the vitamins extracted, owing either to the too low concentration of one vitamin with respect to the others in the extract, as in the case of cyanocobalamin in oral liquid tonics [16], or to the presence of interfering substances in the extracts even after extensive clean-up from complex food matrices, as in the case of almonds, where only two vitamins out of six could be correctly determined [20].

2.2.2. Thin-layer chromatography

Zang and Ma [29] proposed a TLC method to determine thiamine, riboflavin and vitamin B6 simultaneously in various plant materials, using highly sensitive FLD. They claimed that the addition of Triton X-100 to the extractant (*n*-butanol) improved the effectiveness of the extraction media. Shrivastava and Prakash [30] used scolecite (a type of zeolite) in its pure form as an adsorbent for the TLC of a few vitamins.

2.2.3. Micellar electrokinetic capillary chromatography

To date MECC has not been applied to simple or complex food matrices. Nishi *et al.* [31] studied the retention behaviour of eleven water-soluble vitamins in MECC, using a 65 cm × 0.05 mm I.D. fused-silica capillary tube and UV detection at 210 nm, with 0.05 *M* SDS and/or 0.2 *M* sodium lauroylmethyltaurate (LMT) as the anionic surfactant in the electrophoretic medium. To clarify the effect of the micelle, they employed an ion-pairing reagent (sodium pentanesulphonate) that does not form a micelle structure. They separated all solutes within 15 min using a 0.05 *M* SDS solution (pH 9).

Fujiwara *et al.* [32] employed the same surfactant (SDS) and on-column UV detection at 254 nm, with ethyl *p*-aminobenzoate as the internal standard, to determine seven vitamins (thiamine, nicotinamide, nicotinic acid, pyridoxine, cyanocobalamin, L-ascorbic acid and riboflavin phosphate) obtaining DLs of 0.5 pmol for riboflavin phosphate and cyanocobalamin, 1 pmol for thiamine and ascorbic acid and 4 pmol for the other vitamins.

Ong *et al.* [33] separated a mixture consisting of dansylamino acids and two vitamins (riboflavin and pyridoxine) in a single run, using a 60 cm × 50 μm I.D. fused-silica capillary as the separation tube and 40 m*M* SDS in 0.1 *M* borate–0.05 *M* phosphate buffer (pH 7.56) as the electrolytic medium. The detection was carried out with an on-column FLD system with a wavelength programme [0.1 min, 340/400 nm (pyridoxine); 6.1 min, 325/550 nm; 7.1 min, 370/440 nm (riboflavin); and 7.4 min, 325/550 nm] in order to have the maximum sensitivity for each of the species in the mixtures.

2.3. Water-soluble and fat-soluble vitamins

Until now, the simultaneous chromatographic separation of both fat- and water-soluble vitamin groups in a single analysis has not been reported for food matrices; however, recently, a first approach

to this type of determination has been attempted for pharmaceutical products and standard solutions, using fairly new analytical techniques.

For example, to separate fourteen vitamins in drugs, Arai and Hanai [34] used an HPLC–DAD system, equipped with a helium–cadmium laser for fluorescence and a deuterium lamp for absorption detection. They separated the vitamins at 40°C on an Inertsil ODS column with gradient elution from 3% ACN in 0.1% phosphoric acid with 5 m*M* 1-pentanesulphonate to 97% ACN over a period of 20 min, with detection wavelengths ranging from 220 to 400 nm, selecting the maxima for each vitamin. They claimed that the DL with the laser FLD system was about 2000 times less than that with conventional FLD using a photomultiplier. They also stressed that when RP in gradient elution is used, the comparison of retention times is not sufficient for the qualitative analysis of vitamins. They also confirmed that standard ODS columns are not suitable for the determination of some vitamins (such as thiamine and pyridoxamine), owing to peak broadening and absorption phenomena on the packing material. Hence the use of DAD monitoring of fluorescence and absorption spectra and an end-capped ODS column should solve the difficulties with the simultaneous analysis of vitamin mixtures.

Another solution to the problem could be an apparatus consisting of a sample introducer, a switching path connected to a lipid-soluble vitamin trap, positioned between two flow paths (one for each group of vitamins) and the sample introducer, an RP column and a suitable detection system. Mikami [35] using this type of device separated seven water-soluble and five fat-soluble vitamins with a single run.

Another interesting approach to solve this analytical problem could be MECC. Ong *et al.* [36] successfully separated a mixture of seven water- and two fat-soluble vitamins in a single analysis by MECC, using a 50 cm × 50 μm I.D. fused-silica capillary as the separation tube and a micro UV spectrophotometer set at 210 nm as detector. In addition to SDS, they studied the effect of the introduction into the electrophoretic media of modifiers such as γ-cyclodextrin, β-cyclodextrin and 2-propanol on the overall separation of the nine vitamins. Among these modifiers, the combination of 3 m*M* γ-cyclodextrin with 30 m*M* SDS in the electrophoretic medium (0.1 *M* borate–0.05 *M* phosphate, pH 7.6) provided the best selectivity (Fig. 2).

3. FAT-SOLUBLE VITAMINS

3.1. Vitamin A

Vitamin A in its various forms (retinol, retinyl esters, etc.) is present in food of animal origin (*e.g.*, liver, milk, dairy products, fish, poultry, meat), while its precursors (provitamin A) in form of carotenoids are present in both animal and plant foods.

As provitamin A can be biologically converted into vitamin A in the human body, plant foods are an important dietary source of this vitamin, especially for people in developing countries (82% of the total dietary vitamin intake) [37].

3.1.1. Retinoids

The classical spectrophotometric and fluorimetric methods are still official [38], but they cannot discriminate molecular species with different vitamin A activity.

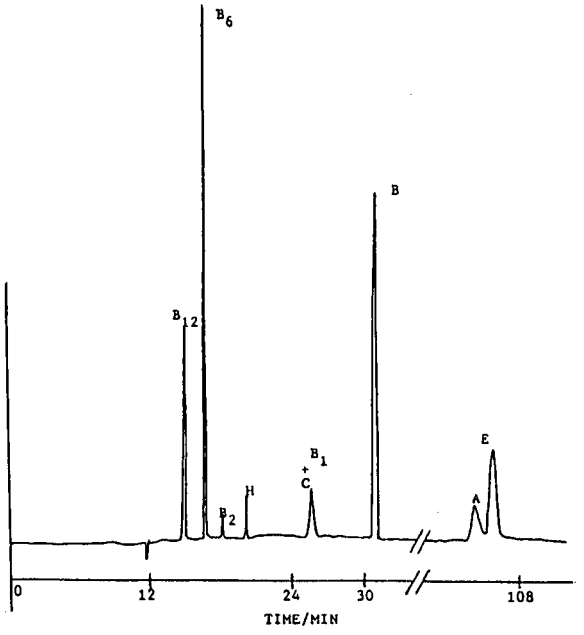

Fig. 2. Electrokinetic chromatogram of the vitamins with 2-propanol. Electrophoretic solution, 30 *M* SDS in 0.1 *M* borate–0.05 *M* phosphate with 3% isopropyl alcohol (pH 7.6); separation tube, 50 cm × 50 μm I.D. fused-silica capillary; voltage, 15 kV; amount injected, 0.75 nl [36].

The naturally occurring compounds having vitamin A activity and the synthetic analogues of retinol, with or without vitamin A activity, are included in the term 'retinoids'. Some retinol metabolites, such as retinal and retinoic acid, play an important role in the human organism.

The problems encountered in the determination of vitamin A in food are similar to those with biological samples. Wyss's review [39] on retinoid chromatography in drug and biological samples pointed out that there had been little progress because of the lack of suitable techniques for the determination of these extremely labile compounds, and stressed the success of HPLC in isolating and separating retinoids and in the identification of new metabolites, improving drug assay. De Leenheer *et al.*'s general review [40] on the fat-soluble vitamins in clinical chemistry pointed out that HPLC was the most applied technique. A review specifically dedicated to food was recently published by Tee and Lim [41], reporting general methods for carotenoids and retinoids.

Retinoids are very sensitive to light and oxidation and they are subject to isomerization and degradation during processing. As a consequence, several processed foods, namely milk infant formula, are fortified with synthetic vitamin A (usually retinyl palmitate) during or after processing. This aspect has to be pointed out because low or not active retinoids are formed.

However, while HPLC is now generally recognized [1] as the best technique for retinoid determination, for official purposes the HPLC methods must be compared with the official ones; for example, Mills [42] compared the Carr–Price colorimetric method of the AOAC [43] and their NP-HPLC method for milk. Thompson [44] discussed the problems with official methods for the determination of vitamin A in food and feeds.

3.1.1.1. Sample preparation. Saponification followed by solvent extraction and direct extraction are the main techniques used to prepare samples for vitamin A analysis (Table 4). Generally, the former is used for total retinol determination according to the AOAC [43] and it was applied, *e.g.*, by Al-Abdulaly and Simpson [45], while many workers [46–50] added an antioxidant such as ascorbic acid, hydroquinone or pyrogallol. Zahar and Smith [51] set up a rapid method where saponification and extraction stages are performed in the same centrifuge tube.

Direct extraction without saponification was used by Woollard and Woollard [52] for HPLC direct injection, by Woollard and Indyk [53] according to a previous method [54] to study the distribution of retinyl esters in milk and by McNeill *et al.* [55] to evaluate the addition of retinyl palmitate to skimmed milk powder as a fortifier during processing. The saponification method allows the determination of free retinoids [45–49,51), whereas the saponification step is omitted when the separation and determination of the different retinyl esters in food are required [52,53,56].

All these methods claim high recovery and efficiency of the selected procedures in preventing isomerization and oxidation of the vitamin.

3.1.1.2. Open-column chromatography. Despite its high specificity and sensitivity, HPLC has the disadvantage of being expensive to set up and maintain, so it is out of reach in most developing countries.

Al-Abdulaly and Simpson [45] developed an inexpensive method using an RP-FC with an open column pressurized with nitrogen and spectrophotometric determination of the eluted vitamin A band. Milk infant formula, margarine, egg yolk, chicken and lamb liver were analysed using this method and compared with the AOAC official method [43] and with an HPLC method [45]. The vitamin A recoveries were 98% with RP-FC, 99% with HPLC and 97% with the AOAC 1984 method. The mean value obtained by RP-FC did not differ significantly from those for the other two methods. The time of analysis of saponified extracts was within 15 min, comparable to that for HPLC, while that for the AOAC method was 35 min, with a lower cost than HPLC.

3.1.1.3. High-performance liquid chromatography. HPLC methods have been developed over the last 5 years according to both the well established modes of RPC and NPC (Table 4). Concerning RP methods, some papers report the application of recommended methods; e.g., Pepping *et al.* [46] used an EEC Cost 91 recommended method [57] for the RP-18 separation of vitamin A in fish oils. RPC on C_{18} was also used by Al-Abdulaly and Simpson [45] for a comparison between HPLC and RP-FC methods, and by Ötles and Hisil [50] for egg analysis, while Coverly and Macrae [47] used a C_8 phase in

TABLE 4

CONDITIONS FOR HPLC DETERMINATION OF RETINOIDS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Milk: saponify with 15% KOH in methanol (70°C, 30 min). Extract with diethyl ether, dissolve in LP. Margarine: dissolve in hexane, filter to dryness, saponify with 80% KOH in methanol 70°C, 30 min), then as above. Egg, liver: dry sample over Na_2SO_4, saponify with 70% KOH, then as above	Open column: 20 × 1.5 cm I.D., 50-μm C_{18}, N_2 (10 p.s.i.) HPLC, RP C_{18}	Water–methanol (10:90), 100 ml ACN–CH_2Cl_2–CH_3OH (70:20:10)	325 nm in LP 325 nm	Milk, infant formula, margarine, egg, chicken and lamb liver	45
Alcoholic saponification (hydroquinone as antioxidant)	C_{18}	Water–methanol (96:4); flow, 1.5 ml/min	294 nm	Fish	46
Automatic continuous Technicon sampling, on-line KOH saponification, clean-up	Supelco LC-8	87% aqueous CH_3OH containing 0.1% CH_3COOH	325 nm	Milk, infant formula	47
Saponify with 10% ethanolic KOH with pyrogallol at room temperature for 18 h in centrifuge tube	Silica Apex	Heptane–2-propanol	DAD	Milk, infant formula	48
Saponify with hot water–alcoholic KOH (30 min) with ascorbic acid as antioxidant, extract with LP	μPorasil	Hexane–$CHCl_3$ (60:40)	Fluorescence, 350/470 nm (ex./em.)	Premixes	49
In a centrifuge tube saponify with KOH (ascorbic acid and pyrogallol as antioxidant, 80°C, 20 min), add LP, shake, evaporate supernatant to dryness, dissolve in methanol	Nova-Pak C_{18}	Methanol–water (95:5); flow, 0.8 ml/min	325 nm 313 nm	Milk, dairy products	51
Saponify with aq. ethanol and ascorbic acid (boil with water, 30 min) extract with hexane, evaporate to dryness, dissolve in ethanol	μBondapak C_{18}	CH_2Cl_2 containing 0.001% trimethylamine–ACN–methanol (300:700:0.5); flow, 0.3 ml/min	280 nm	Eggs	50
Extract with hexane, clean up on PTFE (0.45 μm)	Radial silica	2-Propanol–hexane (0.07:99.93); flow, 2 ml/min	Multi-wavelength UV, fluorescence 325/465 nm (ex./em.)	Vegetable oils, ghee, butter, margarine	52
Milk: dilute with ethanol, extract with LP. Powder: extract with DMSO–DMF–$CHCl_3$ (2:2:1). Oils: dilute with ACN–CH_2Cl_2 (1:1)	Novapak RPC Porasil NPC	ACN–CH_2Cl_2 (80:20) Hexane–2-propanol (99.93:0.07)	325 nm, fluorescence 325/470 nm (ex./em.)	Milk, milk products, shark and cod liver oils	53
Extract from model system with CH_3OH–C_2H_5OH–acetone (6:3:10) and 200 ppm BHT, evaporate to dryness, dissolve in methanol	NPC LiChrosorb Si 60, 45°C RP Spherisorb ODS	Hexane–2-propanol (99.93:0.07); flow, 1.5 ml/min Methanol–water (90:10); flow, 1.5 ml/min	325 nm	Model system	56

an automatic continuous-flow method. The main elution system was aqueous methanol (range 87–96%) but a NARP system, such as with ACN–CH_2Cl_2–MeOH [45], was also used too.

Woollard and Indyk [53], using ACN–CH_2Cl_2 as the eluent, evaluated the distribution of retinyl esters in milk of different animal origin. May and Koo [58] discussed the behaviour of retinyl esters on an ODS column and observed that a plot of log k' *versus* the percentage of water in the eluting solvent was very close to linear in the range 1–3.5% of water in methanol. Measuring the effective carbon number (ECN) is useful for evaluating the effect of the introduction of double bonds into a molecule; some monounsaturated acyl esters have an ECN = 1.85. Hence the resolution of retinyl ester homologues can be evaluated.

NPC has been adopted in many methods using silica gel as a stationary phase, as carried out by Thorpe [49] for total retinol determination and by Thompson and Duval [48] and Woollard and Woollard [52] for the isocratic separation of *cis* isomers and retinyl esters. The latter workers applied a previously tested method [54] demonstrating the formation of several *cis* isomers, which were determined in manufactured UHT milk and milk powders. Skurikhin *et al.* [59] also applied NP in microcolumn HPLC using a 120 × 2 mm I.D. column filled with Silasorb with UV detection to analyse milk and colostrum. Studying the degradation of retinol during storage for low water activity model systems simulating dehydrated food, Manan *et al.* [56] found that the mode of HPLC analysis (NPC or RPC) influence the kinetic interpretation. With NPC the degradation could be adequately described by first-order kinetics, whereas with RPC better results were achieved using a second-order model. This discrepancy was attributed to the formation of a *cis* isomer, which was not resolved from all-*trans*-retinol using RPC; hence NPC showed a better resolution of *cis* isomers. On the other hand, the RPC and NPC modes are equivalent for total vitamin A analysis.

UV detection is widely used, with either fixed- or multi-wavelength instruments, even though DAD was employed by Thompson and Duval [48] and FLD by Thorpe [49]; Woollard and co-workers [52,53] used both modes of detection, and claimed that FLD is preferable because it allowed the visualization of the vitamin A components without the interference of other lyposoluble compounds.

To date, non-aqueous ED for LC analysis of vitamin A in food has not been applied. Hart and Jordan [60] for multivitamin tablets used ED with an inexpensive low-pressure RPC ststem, and Bryan *et al.* [61], studying the use of non-aqueous ED for retinoids after their NP separation on either silica or PVA-sil columns, found a DL of about 1 ng on-column compared with 2 ng with UV detection.

3.1.1.4. Other chromatographic techniques. Retinoids are sensitive to the hot injector port surface and to incompletely inactivated column packings. Smidt *et al.* [62] used cGC to determine underivatized retinol on a bonded-phase methylsilicone column by using a cold on-column injector and FID and claimed a DL of 3.5 ng for retinal. Fürr *et al.* [63] determined the Kováts retention indices of some retinoids for various bonded phases, confirming that the underivatized retinoids can be determined by cGC with on-column injection and either FID or MSD when identification is required. The universal nature and sensitivity of FID and MSD are also advantageous over the more specific detection methods available for LC. Hence this technique can be a valuable tool also in food analysis. So far HPLC–MS has been reserved for the determination of subnanogram amounts of retinoids in clinical chemistry [64].

3.1.2. Carotenoids

Rather than studying quantitatively the provitamin A activity, there have been extensive studies of the qualitative aspects concerning structure elucidation, which is of taxonomic and phytochemical importance. This was pointed out in Rodriguez-Amaya's recent critical review on provitamin A in plant food [65]; she listed four factors that make it difficult to obtain reliable provitamin data: the number of carotenoids present in plant foods; the variability of the qualitative and quantitative composition of food samples; the identification of provitamin A-active compounds and the variations in their activity in different foods; and the formation of artefacts during analysis.

3.1.2.1. Open-column chromatography. Conventional column chromatography (Table 5), often referred to as open-column chromatography, coupled with visible absorption detection has long been used

TABLE 5

OPEN-COLUMN METHODS FOR CAROTENOIDS

Extraction	Column	Eluent	Detector	Food	Ref.
Blend with acetone and Celite, extract with LP and water, saponify with 10% NaOH in methanol overnight at room temperature	MgO–Hyflo Supercel	1–15% acetone in LP	Visible	Vegetables	68
Blend with acetone, extract with LP and water, saponify with 60% KOH in methanol overnight at room temperature under nitrogen	RP-18	Acetonitrile–$CHCl_3$ (92:8), 10 p.s.i., under N_2 flow	Stopped-flow visible spectra	Vegetables	70

in carotenoid research [65]. Most published work, however, deals with the identification of carotenoids, with only a rough estimation of the quantitative composition.

The AOAC [66] and COST 91 [67] quantitative methods for carotenes do not attempt to separate the individual carotenoids, whereas HPLC methods give better separations, identification and quantification.

A modified method for the extraction and determination of individual provitamins used an MgO–Hyflo Supercel column eluted with 1–15% acetone in LP [68]. The results of this method were comparable to those of HPLC methods. To avoid provitamin degradation during hydrolysis of xanthophyll esters, an assessment study by Kimura *et al.* [69] recommended that saponification be carried out overnight at room temperature in LP with an equal volume of 10% methanolic KOH under a nitrogen atmosphere or with an antioxidant. Tsai *et al.* [70] proposed an alternative method based on RP-FC which separates β-carotene from β-cryptoxanthine, but did not succeed in separating β-carotene from lycopene; they claimed more than 97% recovery, calculated by spiking fruits with standards.

3.1.2.2. Thin-layer chromatography. There are few examples of TLC applications for quantitative analysis in the recent literature. Generally, TLC of carotenoids is carried out by NPC on alumina or silica layers eluted with a mixture of hydrocarbon carrier (hexane, LP or benzene) and a polar organic modifier (acetone, chloroform or methanol). The NP method using acetone–LP mixtures for developing silica layers is still used in semi-preparative separations prior to HPLC analysis [71,72].

Francis and Isaksen [73] improved the qualitative separation of oxygenated carotenoids using LP containing tertiary alcohols, which are unaffected by the amounts of lipids found in carotenoid extracts.

3.1.2.3. High-performance liquid chromatography. Numerous reviews have been published over the last few decades, dealing especially with HPLC analysis [41,65,74–77].

Rodriguez-Amaya [65] reviewed the HPLC methods set up to determine the provitamin A content of foods up to 1987, and pointed out the difficulties in separating and determining isomers with a real provitamin activity. NPC (Table 6) has seldom been used in recent years. NPC analyses for carotenes have been developed using a variety of stationary phases. Magnesium oxide and alumina have frequently been used, but active alumina decomposes some carotenoids such as lycopene [78], depending on the retention time in the column and on alumina activities. The *cis* and *trans* isomers of α- and β-carotenes were separated on lime [79]. Silica has been less useful for carotenes, giving long retention times with poor resolution [80]. Rhodes *et al.* [81] suggested using an isocratic mobile phase with a definite water content and silica as stationary phase; they claimed quantitative recoveries and a DL of 20 ng for β-carotene. NPC has been judged inadequate

for carotenoid separations [82]. Although capable of resolving the hydrocarbons, the polar xanthophylls are highly retained on silica unless relatively complex gradient programmes are employed. Further, the carotenoids easily degrade on the slightly acidic surface of silica.

RPC is widely utilized, but carotene separation is difficult and may require the use of gradients or

TABLE 6

CONDITIONS FOR HPLC DETERMINATIONS OF CAROTENOIDS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extract with Na_2SO_4, $MgCO_3$, THF in a Waring blender at moderate speed for 5 min	Partisil 5 ODS	Acetonitrile–THF–water (85:12.5:2.5); flow, 2 ml/min	470 nm	Fruit and vegetables	108
Blend with Na_2CO_3 (pH 8–9) and acetone (10 min, 0–5°C) extract with diethyl ether and water, TLC on silica layer developed with LP–acetone (1:1)	Hypersil ODS	(A) Methanol–water (75:25) (B) ethyl acetate. Gradient: 0 min, 100% A; 10 min, 70% B; 14 min, 100% B; flow, 1.7 ml/min	430 nm	Kiwi	72
Extract with CCl_4–methanol (2:1)	Chromsil C_{18}	Acetonitrile–2-propanol–water (39:57:4); flow, 1 ml/min	438 nm	Tomato, paprika	86
Saponify with ethanolic KOH in the presence of ascorbic acid, extract with diisopropyl ether	Hypersil ODS	Methanol–water–acetonitrile–$CHCl_3$ (200:11:250:50); flow, 1.5 ml/min	445 nm	Vegetables	109,110
Extract with DMF, filter, extract with hexane	Spherisorb ODS-2	Gradient from A to B. A = water–IP reagent–methanol (1:1:8) [IP = 0.05 *M* tetrabutylammonium acetate –CH_3COONH_4, 1 *M* in water]. B = acetone–methanol (1:1)	DAD	Olive	71
Extract at 100°C with hexane–acetone–diethyl ether (30:50:20)	ODS RP-18, 22°C	CH_2Cl_2–methanol–acetonitrile (10:27:63); flow, 1 ml/min	DAD	Dried carrots	100
Saponify with 50% KOH in ethanol and 0.1% ascorbic acid, extract with LP–diisopropyl ether (75:25) and water	RP C_{18}	Acetonitrile–CH_2Cl_2–methanol–2-octanol (70:20:10:0.1); flow programme, 0.7–3.5 ml/min	436 nm 313 nm	Milk	112
Blend with acetone–hexane (40:60), column chromatography on deactivated alumina	ODS C_{18}	Methanol–ACN–$CHCl_3$ (47:47:6); flow, 2 ml/min	461 nm	Raw and cooked vegetables	111

TABLE 6 (*continued*)

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extract with acetone, Na_2SO_4, $MgCO_3$, saponify with KOH in ethanol and water overnight at room temperature, extract with hexane–diethyl ether (70:30) with BHT (0.1% in hexane)	Zorbax ODS	ACN–CH_2Cl_2–methanol (70:20:10); flow, 2 ml/min	450 nm	Vegetables, fruits, berries	113
	Spherisorb ODS-2, 30°C	ACN–methanol–CH_2Cl_2–hexane; (A) 75:15:5:5; (B) 40:15:22.5:22.5. Gradient: 0–6 min A, 6–20 min A to B; flow, 1 ml/min			
Dissolve in CH_2Cl_2, saponify with KOH–methanol extract with LP	Rainin Microsorb ODS	ACN–methanol–CH_2Cl_2 (60:35:5); flow, 2 ml/min	DAD	Palm oil	99
Blend with H_4folic acid, Na_2SO_4, $MgCO_3$ and I.S, extract with LP–water, saponify with 30% KOH–methanol, purify on C_{18} TLC plates	Microsorb C_{18}	Methanol–ACN–CH_2Cl_2–hexane (1:1): (A) 10:85:5; (B) 10:45:45. Gradient: 0–10 min A, 10–40 min A to B; flow, 0.7 ml/min	DAD	Fruit and vegetables	91
Extract with CS_2–acetone–hexane (2:2:1), saponify with ethanol–KOH solution	Supelcosil LC 18 S	(A) 2-propanol–1,2-dichloroethane (2:1); (B) acetonitrile. Gradient: 0 min, 5% A; 10 min, 7.5% A; 15 min, 30% A; 30 min, 50% A; flow, 1.5 ml/min	458 nm	Shellfish	107
Blend with acetone–ethanol (1:1), extract with LP without saponification	$Ca(OH)_2$	Hexane–acetone (99:1); flow, 0.25 ml/min	DAD	Vegetables	79

complex ternary or even quaternary mixtures. There are some disadvantages of RP analyses, such as long run times and the use of less volatile solvents, which make solvent removal difficult in preparative work. C_{18} column selection must be carefully chosen, as the performance varies considerably from batch to batch.

RP separations with water are still in use according to Schwartz *et al.* [83] and Bushway and Wilson [84]; the addition of water greatly improved the resolution of the xanthophylls and revealed a greater complexity in the mixtures of these components. On the other hand, the retention times of all compounds increased up to 300 min for carotenes [85]. Biacs and co-workers [86–88] conducted some studies on mobile phase composition and claimed that using ACN instead of acetone prevents column deterioration and sample degradation [88].

Nelis and De Leenheer [89] proposed NARP chromatography as a useful approach for the isocratic separation of non-polar compounds. In order to cover the whole range of polar and non-polar carotenoids, many systems employ gradient elution (Table 6).

The choice of the right C_{18} phase has been widely investigated. Khachik and co-workers [90,91] compared Rainin Microsorb C_{18} (with a high carbon loading and small pore size) and Vydac C_{18} (with a

low carbon loading and a large pore size); the latter gave shorter retention times. A qualitative study showed that, among the various classes of carotenoids, the separation of the stereoisomers of the hydrocarbon carotenoids could be better accomplished on a Vydac column; on the other hand, the separation of the oxygenated carotenoids (xanthophylls and their stereoisomers) was better on a Microsorb C_{18} column. The same group [92] developed a method to separate and identify fatty acid esters which have different fatty acid side-chains. Khachik *et al.* [93] were concerned about possible artifacts and peak distortion caused by the different solubilities of carotenes in the injection solvent (THF and CH_2Cl_2) with respect to a mobile phase containing a polar modifier.

Recently, Epler *et al.* [94] evaluated 65 HPLC columns under standardized conditions for carotenoid separation and recovery. They agreed with Quackenbush's [95] observations that polymeric C_{18} phases showed excellent selectivity of structurally similar carotenoids, whereas the most commonly used monomeric C_{18} phases did not. Epler *et al.* found that mobile phases containing ACN gave lower recoveries than those containing methanol.

From optimization studies on isocratic separations using a polymeric C_{18} column, the best separation in the shortest analysis time was achieved by using THF as modifier in the methanolic eluent [96] (Fig. 3). Sander and Craft [97] gave an example of enhanced shape discrimination at sub-ambient temperatures, separating 9-*cis*- and-*trans*-carotenoids at -13°C.

Granado *et al.* [98] compared monofunctional and polyfunctional C_{18} columns with a series of vegetable samples; they observed a marked increase in the resolution, especially for polar carotenoids, and a decrease in the separation factors. They reported DLs ranging from 0.1 ng for canthaxanthin to 2.4 ng for phytoene using dual-channel dispersive UV detection.

Ng and Tan [99] developed a NARP–DAD method to identify palm oil carotenoids; they found that the β-carotene DL was 31 ng, confirming that DAD has a lower sensitivity than fixed-wavelength monochromator detectors currently being used; on the other hand, DAD is necessary for identifying and characterizing the various carotenoid isomers.

Lesellier *et al.* [100] developed NARP chromatography to optimize the separation of *trans*- and *cis*- α- and -β-carotenes, studying the effects of the elution strength of the solvents, their solubility properties, the influence of temperature on the capacity factor and selectivity. They stressed the need for a polymeric bonding on the stationary phase to separate *trans–cis* isomers. The same group [101,102] assessed an SFC method with a C_{18} column and CO_2 containing a polar modifier; this supercritical fluid has a solvent polarity similar to that of hexane, but it has a lower viscosity; therefore, solute diffusion coefficients are higher than in conventional solvents. The relationships between capacity factors and operating parameters such as

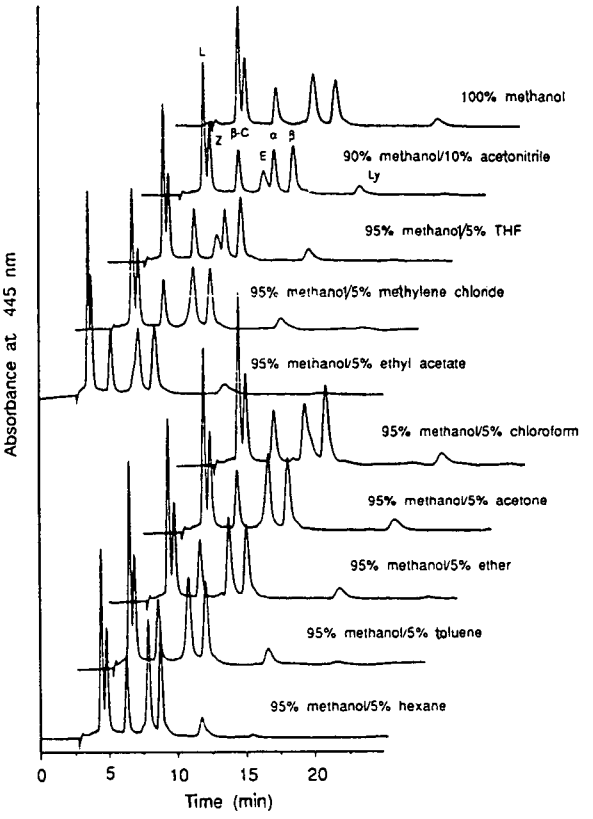

Fig. 3. Effect of nine solvent modifiers on the separation of seven carotenoids. Chromatographic conditions: Vydac 201TP C_{18} (5 μm) column (250 × 4.6 mm I.D.); mobile phases as shown; flow-rate, 1 ml/min; UV–VIS detection at 445 nm; column temperature, 25°C. Peaks: L = lutein; Z = zeaxanthin; β-C = β-cryptoxanthin; E = echinenone; α = α-carotene; β = β-carotene; Ly = lycopene [96].

temperature, pressure, nature and concentration of the polar modifier were investigated. These studies have led to a fivefold reduction in the analysis time together with an improvement in the resolution of these compounds.

An interesting study by O'Neil *et al.* [103] compared methods for the determination of *cis–trans* isomers of β-carotene. Although both Vydac C_{18} and $Ca(OH)_2$ were effective in resolving diastereoisomers of β-carotene, they found that the lime method had fewer interferences and higher selectivity when used to analyse real vegetable samples. The only possible way to separate a complex mixture of esters and carotenes is the use of an NP and an RP in series: on NP, cryptoxanthin esters are well separated from the diesters. NP provides quantitative data on the different classes of carotenoids and RP determines the individual elements.

The choice of the correct method for quantitative analyses was discussed by Philip and Chen [104]; owing to the limited availability of pure carotenoids as standards, the use of external standards is rare, whereas the use of an I.S. is affected by the shifts in absorption maxima in different solvents during gradient elution. They claimed that Sudan I is suitable as an I.S. for both NP and RP carotenoid analysis.

There are other techniques besides NP and RP chromatography, but they are less frequently used. Minguez-Mosquera *et al.* [71] presented a method using IP-RP chromatography to study olive fruit lactic fermentation; pigment concentrations were evaluated from an extension of Beer's law. This procedure was compared with the external standard method; an analysis of variance showed no significant differences between the two methods.

Stalcup *et al.* [105] proposed a cyclodextrin bonded phase used in the NP mode to separate and identify a complex standard mixture. An improvement in the identification and characterization of various carotenoids could be the use of MSD in LC analysis. This topic was reviewed by Taylor *et al.* [106].

3.2. *Vitamin D*

HPLC is the most commonly used chromatographic method for the determination of vitamin D vitamers [ergocalciferol (D_2) and cholecalciferol (D_3)], which have very similar biological activity in man. Usually, total vitamin D evaluation is required, even if the knowledge of the content of each vitamer in fortified food is important.

Although in the past other techniques, such as open-column chromatography, TLC and GC, were applied [114], over the last 5 years these techniques have not been improved. In contrast, HPLC techniques have been extensively studied over the last decade [1] and two methods, which were tested by collaborative trials by de Vries and co-workers [115,116], were accepted as official by the AOAC [117,118].

3.2.1. *Sample preparation*

Usually, sample preparation is carried out in two steps: saponification and clean-up (Table 7). The most commonly applied mode is saponification with refluxing alcoholic KOH and it was included in AOAC methods, with some precautions to reduce thermal isomerization [119–122] and to prevent oxidation by adding some antioxidants [117,118,123–126]. Agarwal [127] used an overnight cold saponification, while Hung [126] did not saponify the samples before extraction.

The clean-up stage is the critical point of the methods and it appears rather complex. This is still a matter of major development, as a well prepurified extract is needed for analytical HPLC. To avoid these clean-up procedures, Agarwal [127] converted both vitamers present in the unsaponifiable matter from fortified milks into isotachisterol by reaction with $SbCl_3$; the derivative was measured at 301 nm without interference from the other substances present.

The clean-up procedure varies according to the type of food matrix. For example, for fortified milk and milk powders [117], semi-preparative HPLC on a CN-bonded phase prior to the analytical HPLC is suggested, whereas for feedstuffs [118] a two-step clean-up is necessary, involving open-column chromatography on alumina to separate vitamin D from vitamin E and carotenoids, followed by purification of the vitamin D fraction by semi-preparative HPLC on an RP-18 column, prior to the analytical HPLC.

Clean-up by open-column chromatography on alumina was also used by Villalobos *et al.* [122] for corn flakes and sardines and by Romanov and Osipova [121] on plant materials. Bekhof and Van den Bedem [119], using a two-dimensional HPLC sys-

TABLE 7

SAMPLE PREPARATION FOR HPLC DETERMINATION OF VITAMIN D

For analytical HPLC conditions see Table 8.

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Saponify in ethanol–KOH extract with diethyl ether, (1) open-column clean-up, eluate evaporation; (2) semi-preparative HPLC, evaporate solvent, dissolve in hexane, analytical HPLC	*(1) Open column*		Various foods and feeds	Total vit. D	118
	Al_2O_3	Diethyl ether–hexane (40:60)			
	(2) Semi-preparative HPLC				
	Lichrosorb RP-18	ACN–methanol–water (50:50:5); flow, 1 ml/min			
Extract with CH_2Cl_2–BHT–Na_3PO_4, shake for 1 h, filter, evaporate, dissolve and 4-step clean-up	*(1) Sep-pak*		Various feeds	Vit. D_2, D_3	126
	Silica	1% Ethyl acetate in benzene, evaporate, dissolve in CH_2Cl_2			
	(2) Filtration				
	Teflon F.H.O., 5 μm	Evaporate, dissolve in $CHCl_3$			
	(3) GPC				
	Sephadex LH-20	$CHCl_3$–isooctane (98:2), collect fraction at 29–56 min, evaporate, dissolve in CH_2Cl_2			
	(4) Preparative HPLC				
	Partisil 10 PAC	Methanol–water (90:10)			
Saponify in ethanol–KOH (water-bath, 30 min), extract with pentane. light coloured extracts: direct injection; coloured extracts, open column clean-up, evaporate and dissolve in hexane (column A), mobile phase (column B), methanol (column C)	*Open column*		Corn flakes, sardines	Vit. D_2, D_3	122
	Al_2O_3 neutral	4% Acetone in hexane; 15% acetone in hexane to elute vit. D + vit. A band with fluorescence monitoring			
Saponify in ethanol–KOH (pyrogallol, 80°C, 30 min), extract with benzene, evaporate, dissolve in methanol–ACN (1:1), HPLC	*HPLC*		Mushrooms	Vit. D_2	123
	Lichrosorb RP-18	Methanol–ACN (1:1)			
Saponify in ethanol–KOH (pyrogallol, 80°C, 30 min), extract with benzene, (1) TLC clean-up, $CHCl_3$ extract, (2) HPLC clean-up, HPLC analysis, (3) identification by MS	*(1) TLC*		Mushrooms	Vit. D_2 identification	124
	Silica gel	Benzene–acetone (95:5)			
	(2) HPLC				
	Lichrosorb RP-18	Methanol–ACN (1:1)			

TABLE 7 (*continued*)

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Saponify in ethanol–KOH (pyrogallol, overnight, room temperature), extract with hexane, evaporate, dissolve in $CHCl_3$, isomerization with $SbCl_3$ in $CHCl_3$	No clean-up		Milks	Total vit. D	127
Saponify, extract with benzene, HPLC clean-up, collect vit. D fraction, analytical HPLC	*HPLC* Nucleosil 5-C_{18}	ACN–methanol (3:2)	Baby foods	Vit. D_2	120
Saponify in ethanol–KOH extract with hexane	*Open column* Al_2O_3	2-Propanol–hexane	Plant materials	Vit. D_2	121
Saponify in ethanol–KOH (Na ascorbate, water-bath, 40 min) extract with pentane–BHT, evaporate to dryness, dissolve in the semi-preparative HPLC mobile phase	*HPLC* Si-60 D 10-CN	0.35% Pentanol in hexane, collect vit. D fraction, add BHT, dilute with 5% toluene in hexane	Milk	Vit. D	117
Saponify in ethanol–KOH (water-bath under N_2, + BHT, 30 min) extract in diethyl ether, evaporate, dissolve in methanol, semi-preparative HPLC, collect 24–32-min fraction, dry, dissolve in hexane	*Semi-preparative HPLC* LiChrosorb RP-8, 7 μm	Methanol–water (90:10), detection at 264 nm	Feeds	Vit. D	125

tem, carried out the clean-up on a CN-bonded phase, which was directly coupled to the analytical column via a six-way valve. Preparative RP-18 HPLC was used to separate the vitamin D fraction from the unsaponifiable matter of baby foods [120] and from mushrooms [123], while Laffi [125] used an RP-8 column for feeds. Takamura *et al.* [124], in order to identify vitamin D_2 in mushrooms by HPLC–thermospray MS, carried out a preparative TLC prior to the preparative RP-HPLC step.

When the saponification step is omitted to prevent isomerization, a more extensive clean-up procedure is necessary [128]. Hung [126] carried out a four-step clean-up procedure: a silica cartridge clean-up, followed by filtration through a Teflon membrane and then by GPC and semi-preparative NP-HPLC. This procedure was claimed to be especially useful for determining low levels of vitamin D_2 and D_3 in complex matrices.

3.2.2. High-performance liquid chromatography

Both NPC [117,118,120,121,125,127] and RPC [126] have been used to determine total vitamin D (Table 8). The results obtained with AOAC official methods [117,118] by NPC have to be corrected for the effect of isomerization due to the high temperature applied during the saponification stage (factor: 1.25). However, vitamin D_2 and D_3 are resolved only by using RPC [126]; in fact, comparing the separation efficiency for vitamin D_2 and D_3 for three types of packing materials, using eight selected types of matrix after saponification and the

AOAC clean-up procedure [117,118], Villalobos *et al.* [122] found that RP columns separate vitamin D_2 and D_3, whereas an NP silica column does not resolve the two vitamers. Bekhof and van den Bedem [119] detected simultaneously the two vitamers using an NH_2-bonded phase. Generally a UV detector was used, either at a fixed wavelength of 254 nm or with multi-wavelength models set at 260 nm [119] or at 301 nm, as carried out by Agarwal [127] in an isomerization method.

Takamura *et al.* [124] used MSD to identify vitamin D_2 in mushrooms. The choice of HPLC mode (RP or NP) depends on the aim of the analysis: for total vitamin D content NP is very efficient, whereas to separate vitamin D_2 from D_3 an RP phase is necessary, although an NH_2-bonded phase is re-

TABLE 8

CONDITIONS FOR HPLC ANALYTICAL DETERMINATION OF VITAMIN D

For sample preparation see Table 7.

Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
5-μm Partisil 5	0.35% Pentanol in hexane	UV 254 nm	Various foods and feeds	Total Vit. D	118
3 *M* C_{18} Rainin Accupack Microsorb		UV 254 nm	Feeds	Vit. D_2, D_3	126
(A) LiChrosorb Si 60, 5 μm	Hexane–2-propanol (98.3:1.7); flow, 1.5 ml/min	UV 254 nm	Corn flakes, sardines	Vit. D_2, D_3	122
(B) Vydac 201 TPB C_{18}, 10 μm	Methanol–$CHCl_3$–ethyl acetate (88:4:8); flow, 1.5 ml/min				
(C) Baker ODS, 5 μm	Methanol–water (98:2); flow, 1 ml/min				
Nucleosil 100-5, 5 μm	Hexane–1-pentanol–2-propanol (99.5:0.1:0.4); flow, 1 ml/min	UV 254 nm	Mushrooms	Vit. D_2	123
Nucleosil 100-5, 5 μm	Hexane–1-pentanol–2-propanol (99.5:0.1:0.4); flow, 1 ml/min	UV 254 nm, dissolve in CH_3COONH_4–methanol, thermospray MS	Mushrooms	Vit. D_2 identification	124
Silica Spherisorb, 3 μm	Hexane–ethyl acetate–methanol (97:2.5:0.5); flow, 0.7 ml/min	UV 301 nm	Milks	Vit. D	127
Zorbax Sil	2-Propanol–hexane (0.8:99.2)	UV	Baby foods	Vit. D_2	120
Silica gel	Hexane–$CHCl_3$	UV	Plant materials	Vit. D_2	121
5-μm Partisil	0.35% 1-Pentanol in hexane	UV 254 nm	Milk	Vit. D	117
LiChrosorb Si 60, 5 μm	7% Dioxane in hexane; flow, 1.0 ml/min	UV 264 nm	Feeds	Vit. D	125

ported also to be efficient in resolving the two vitamers.

All methods claimed a high recovery and high sensitivity for the different samples tested.

3.3. Vitamin E

Vitamin E is an essential nutrient and it is also added to some foods as an antioxidant. There are eight naturally occurring vitamin E vitamers: four tocopherols (α-, β-, γ- and δ-TOC) and the corresponding tocotrienols. They each have a different biological activity, with α-TOC being the most active and α-TOC and δ-TOC being the predominant forms occurring in foods.

Analysis for vitamin E in foods involves separation and individual determination of all the tocopherols, tocotrienols and, if supplemented, tocopheryl acetate. To measure the total vitamin E activity, it is not sufficient to determine α-TOC, as other tocopherols and tocotrienols, such as γ-TOC in vegetable oils and β-TOC in wheat bran, may be present in large amounts and can contribute significantly. In many methods β- and γ-TOC are measured as a combined fraction, whereas the tocotrienols are excluded completely.

Recent reviews [129–131] have discussed various methods for analysing vitamin E isomers, ranging from the spectrophotometric methods of Emmerie and Engel [286] to TLC, GC and HPLC. The last technique has predominated over the last few years, especially as specific detection methods can be employed and exposure to air and handling of the tocopherols can be minimized.

3.3.1. Gas chromatography

GC has long been a useful approach for the qualitative and quantitative determination of tocopherols in natural fats (Table 9). Tocopherols and sterols are found in the unsaponifiable fraction and both have a hydroxyl group, so they are separable by GC under similar conditions. Their GC analysis has been extensively studied, although few papers have been published recently.

Ulberth [132], comparing derivatization systems such as silylation and esterification, claimed that the use of heptafluorobutyril (HFB) esters gave an optimum separation between cholesterol and α-TOC with a capillary column and ECD and proposed this method as an alternative to HPLC for the determination of the vitamin E content in foods of animal origin.

A collaborative study [133] evaluated the feasibility of GC procedures for assaying α-tocopheryl acetate in supplemental vitamin E concentrate. The GC method was more specific and precise, because it separates α-tocopheryl acetate and α-TOC, whereas spectrophotometric methods measure total saponifiable isomers of vitamin E. This method was accepted as official by the AOAC in 1990 [134].

3.3.2. Thin-layer chromatography

TLC methods are usually employed for sample purification [135]. A comparative study [136] on the determination of tocopherols between direct spectrophotometric analysis and TLC with UV detection at 295 nm showed some differences in the results, especially for low tocopherol concentrations. Askinazi *et al.* [137] compared a modified TLC

TABLE 9

CONDITIONS FOR GC DETERMINATION OF TOCOPHEROLS

Compound	Detector	Column	Stationary phase	Solid support	Temperatures (°C)			Internal standard	Ref.
					Injector	Column	Detector		
α-Tocopheryl acetate	FID	2 m (glass)	SE-30	80–100 mesh	275	265	275	Hexadecyl palmitate	133,134
α-TOC, β-TOC, γ-TOC, δ-TOC Cholesterol	FID	20 m × 0.25 mm I.D. (silica)	DB-5	–	300	260 (60 s), 4°C/min to 300	300	Cholestane	132

TABLE 10

CONDITIONS FOR ANALYTICAL HPLC DETERMINATION OF VITAMIN E

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
Saponify with ethanol–KOH (26°C, 10 min, pyrogallol), dilute with water, extract with diethyl ether, wash organic layer with dilute HCl, evaporate to dryness, dissolve in hexane	Silica	2-Propanol–hexane (0.5:99.5); flow, 1 ml/min	Fluorescence, 290/330 nm (ex./em.); UV 292 nm	Vegetable oils, fats	All tocopherols, tocotrienols	148
Extract according to AOAC (1984) method, dilute with hexane–isopropanol (1:4)	Nucleosil 120-5 C_{18}, 5 μm	Gradient elution: (A) water–H_3PO_4, pH 3; (B) ACN–methanol (7:5); (C) 2-propanol; flow, 1 ml/min	UV, 215 nm UV, 280 nm	Sunflower oil	Phenolic antioxidants, tocopherols, triglycerides	131
Saponify with ethanol–KOH (reflux, 30 min, Na ascorbate) extract with LP, evaporate to dryness, dissolve in hexane	Partisil 5-Si, LiChrosorb Si-60	Isooctane–2-propanol (99:1); flow, 1 ml/min	Fluorescence, 293/326 nm (ex./em.)	Animal feeds	α-TOC	149
Saponify overnight with KOH, extract with hexane	Zorbax ODS, 5 μm	ACN–CH_2Cl_2–methanol (70:25:5)	Fluorescence, 290/330 nm (ex./em.)	40 food products	α-,δ-,γ-TOC	154
For RPC: dissolve in ethyl acetate. For NPC: dissolve in hexane. Chill overnight at −20°C, centrifuge at 4°C	Zorbax Sil Zorbax ODS	Hexane–2-propanol (99:1); flow, 2 ml/min ACN–methanol–CH_2Cl_2 (60:35:5); flow, 2 ml/min	DAD	Crude palm oil	All tocopherols, tocotrienols	147

Saponify in ethanol–KOH (pyrogallol, 8 min, 70°C), extract with hexane–diisopropyl ether (3:1), centrifuge, evaporate, dissolve in hexane	Radial Pak C_{18}, 5 μm	Methanol; flow, 1. ml/min	UV, 210 nm; Fluorescence, 295/330 nm (ex./em.)	Various foods	α-TOC, β-+γ-TOC, δ-TOC, cholesterol, phytosterol	139,146
Saponify with ethanol–KOH (30°C, overnight, under N_2), extract with hexane, evaporate to dryness, dissolve in methanol	Biosil ODS 5S	Methanol; flow, 1 ml/min	Fluorescence, 296/330 nm (ex./em.)	Chicken, meat	α-TOC, γ-TOC	155
Fully automatic extraction apparatus	LiChrosorb RP-18, 7 μm	Methanol; flow, 2 ml/min	UV, 288 nm	Tablets	α-TOC acetate	166
Extract fat according to AOAC (1984), saponify with ethanol–KOH and pyrogallol, extract with hexane	LiChrospher Si 60	1% 2-Propanol and 0.5% ethanol in hexane; flow, 1 ml/min	Fluorescence, 292/320 nm (ex./em.)	Infant formula	All tocopherols	156
Dilute with mobile phase	LiChrospher Si 100	Cyclohexane–H_4folic acid (95:5); flow, 1.8 ml/min	UV, 295 nm	Olive oil	α-TOC, γ-TOC	157
Extract with $CHCl_3$–methanol (2:1), saponify with ethanol–KOH (30 min, 70°C), extract with NaCl solution and ethyl acetate–hexane (1:9)	Nucleosil 5-NH_2	Hexane–2-propanol (98.5:1.5); flow, 1 ml/min	Fluorescence, 295/325 nm (ex./em.)	Cereals legumes	All tocopherols	158

corrected 17 June. 93/AP
In third column, second row from bottom:
"Cyclohexane – H_4 folic acid" should read "Cyclohexane – THE"

method for the determination of tocopherol vitamers with GC; both techniques gave consistent results.

3.3.3. High-performance liquid chromatography

3.3.3.1. Sample preparation. Tocopherols in animal and vegetable oils are directly determined after appropriate dilution of the sample with *n*-hexane. Andrikopoulos *et al.* [138] used 2-propanol–hexane (4:1) to prepare oil solutions. Other foods and animal feeds need a previous saponification step under a nitrogen flow. Indyk [139] described a simplified approach to saponification, which involves a single piece of glassware; the tediousness of conventional multi-step saponification was therefore overcome, sample preparation being completed within a single test-tube and the full procedure within 45 min.

Many factors affect the extraction ratio of tocopherols from the saponification medium, such as ethanol concentration in the medium, extraction solvents and co-existing fats. Ueda and Igarashi [140,141] surveyed the factors influencing *n*-hexane extraction from a saponified medium in food and biological samples. Takeyama *et al.* [142] and Ujie *et al.* [143] proposed *n*-hexane instead of diethyl ether as the extraction solvent, because the former is less volatile and contains fewer contaminating compounds. They found an improvement in the extraction ratio of tocopherols with the addition of up to 10% of ethyl acetate in hexane.

Håkansson *et al.* [144] proposed direct hexane extraction from wheat products using a Soxhlet apparatus.

Rizzolo *et al.* [145] evaluated the effect of the extraction technique on almonds, comparing overnight cold oil extraction under a nitrogen atmosphere with Soxhlet extraction followed by either dilution with mobile phase or cold saponification. They found that the cold extraction provided the best accuracy and precision for α- and γ-TOC, whereas it was not suitable for determining the small amounts of β-TOC.

3.3.3.2. Analytical high-performance liquid chromatography. Ball's review [1] covered HPLC methods up to 1987. There have not been many HPLC separations of just tocotrienols and tocotrienols with tocopherols. Methods from 1988 have reported in Table 10, where it can be seen that both NPC and RPC are commonly used.

The acknowledged stability and robustness of the ODS phases are considered to be advantageous for routine application; moreover, RPC is more efficient and versatile when we have to determine tocopherols with other lipids in the same run [138,146]. Unfortunately, the positional isomers β- and γ-TOC cannot be resolved, even if complex gradient elution is employed.

Tan and Brzuskiewicz [147] described an optimization study of seven tocopherol and tocotrienol standards using both NP and RP columns with various isocratic mobile phases. They compared C_{18} columns used in NARP chromatography, but none could separate the two positional isomers; on RP columns the less polar but more saturated tocopherols were retained in the stationary phase longer. NP columns provided separation based on the number and position of methyl substituents on the chromanol moiety, and succeeded in separating positional isomers. The study also considered cyano- and amino-bonded phases; the former was ineffective in the separation of all isomers, but the latter were comparable to silica columns in selectivity and resolution. In addition to the difficulty of separating the eight isomers, these compounds also have similar UV spectra; to characterize the isomers the use of DAD was proposed, with spectra recorded in second- and fourth-derivative form.

NPC allows the complete separation of all eight isomers; these methods were proposed as official methods of analysis from two comparative studies carried out by the IUPAC Commission on Oil, Fats and Derivatives [148] and by the Analytical Methods Committee of the Royal Society of Chemistry [149]. The IUPAC Commission examined also the potency of a Partisil PAC (amino- and cyano-bonded) column; this column is reported as acting as a highly polar stationary phase when used with a hydrophobic mobile phase [hexane–THF (9:1) was the proposed mixture for tocopherols analysis]. All of them achieved good results using this column and the Commission concluded that it was undoubtedly worth investigating the applications of this type of column when it becomes more widely available.

Both interlaboratory studies used FLD,because it allows selective and sensitive monitoring, without interference from artifacts, which can be a problem in some foods when UV detection alone is used.

Moreover, UV detection is at least ten times less sensitive then FLD, and needs an accurate choice of mobile phase composition and column packing material, because UV spectra are sensitive to environmental conditions. Using FLD, α-TOC is appropriate as the sole standard for the vitamin E congeners, as the fluorescence responses are essentially equivalent [139]. The only disadvantage is that, as esters of tocopherols are weakly fluorescent, fortified formulas containing tocopheryl acetate must be subjected to saponification before analysis, with possible degradation of vitamers.

An alternative to FLD could be ED, which needs aqueous RPC, as the supporting electrolyte is soluble in a semi-aqueous mobile phase. Ueda and Igarashi [150] evaluated ED for the determination of tocopherols in feeds; they found that the sensitivity was twenty times higher than with FLD and the DL for α-TOC by ED was 0.1 ng. Luscombe and Bond [151] claimed a DL for α-, γ- and δ-TOC down to 10^{-7} *M* using a surface-modified platinum microelectrode as detector in RPC analysis [methanol–water (95:5)]. MS can be used to allow the unequivocal characterization and determination of tocochromanols, both for detection in HPLC and as a direct analytical technique such as tandem MS, so minimizing the losses due to each manipulative stage [152].

Tri Wahyuni and Jinno [153] investigated the separation of tocopherols on various chemically bonded phases in microcolumn liquid chromatography. For NPC a naphthylethyl-bonded phase column with 0.1% hexafluoropropanol in *n*-hexane as the mobile phase gave the best separation. A polymeric C_{18} column with 8.5% *n*-hexane in ACN as the mobile phase gave the best results in RPC, the four tocopherols being well separated; microcolumn RPC could be developed as an optimum separation method in tocopherol analysis.

3.3.3.3. Preparative high-performance liquid chromatography. It is of commercial interest to isolate tocopherol isomers with a purity greater than 90%. Bruns *et al.* [159,160] achieved a direct scale-up from the analytical to the preparative mode by only a slight change in chromatographic conditions and isolated up to 4 g of 95.4% pure natural D-γ-TOC from 15 g of a vegetable oil extract injected each run (Table 11).

3.3.4. Supercritical fluid extraction and chromatography

SFE with CO_2 is now used in industry as a clean up, solvent-free separation process. A fundamental study was carried out to develop an extraction process for concentrating 18% tocopherols from soybean sludge using supercritical CO_2 [162]. Saito and co-workers [163,164] proposed directly coupled SFE–preparative SFC as a powerful method not only for analytical purposes but also for preparative separation. They achieved the isolation of tocopherols from wheat germ oil by recycling semi-preparative SFC using two 250 × 10 mm I.D. columns packed with 5-μm silica gel. This method has the potential capability of replacing conventional preparative HPLC because it effects extraction, preconcentration and chromatographic fractionation in a single run. The use of supercritical CO_2 allows the easy separation of solutes at a low temperature and in an oxygen-free environment, which is essential for separating labile compounds such as tocopherols. In addition, a non-flammable, non-polluting and inexpensive mobile phase is very helpful for safety and economy in the laboratory and in the production process.

Takeuchi and Saito [165] presented an example of the separation and identification of α- and β-TOC standards using on-line coupling of SFC to FAB-MS with a micropacked ODS column.

3.4. Vitamin K

Three forms of vitamin K vitamers are present in food: vitamin K_1 (phylloquinone, PK) of plant biosynthetic origin, vitamin K_2 (menaquinones, MK), a group of vitamers synthetized by bacteria ranging from MK-4 to MK-13, according to the number of isoprene units in the side-chain; and synthetic vitamin K_3 (menadione MD) and its water-soluble hydrogensulphite derivative (MSB). These compounds are very sensitive to light and are easily reduced to the hydroquinone forms. On this reactivity are based a number of chemical methods [167]. According to published reviews [167–169], there have been great developments in chromatographic techniques for vitamin K analysis in recent decades. However, whereas open-column methods, TLC and GC were widely applied until the 1980s [167,170], more recently HPLC has become more important

TABLE 11

CONDITIONS FOR PREPARATIVE HPLC SEPARATION OF VITAMIN E

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Analyte	Ref.
Purify by silica open column, eluting with hexane	LiChroprep Si 60, 25–40μm	Hexane–butyl methyl ether (97:3); flow, 450 ml/min	UV, 205 nm	Vegetable oils	α-TOC	159,160
Purify by silica open column, eluting with hexane–ethyl acetate (85:15)	HP Si 100	2–10% Ethyl acetate in hexane	UV, 254 nm	Palm fatty acid distillate	Tocotrienol	161

TABLE 12

SAMPLE PREPARATION CONDITIONS FOR HPLC DETERMINATION OF VITAMIN K

For analytical HPLC conditions see Table 13.

Extraction	Chromatographic clean-up		Food	Analyte	Ref.
	Column	Mobile phase			
Lipase treatment, extract with pentane, evaporate, dissolve in 2-propanol, semi-preparative HPLC, evaporate PK and MK-*n* fractions, dissolve in methanol, analytical HPLC	Nucleosil C_{18}, 5 μm, 35°C	Methanol–ACN (1:1), detection at 248 nm	Milks	PK, MK-4, MK-6, MK-7, MK-8, MK-9	171
Extract with $CHCl_3$ and aq.NH_3, neutralize with CH_3COOH, add Celite and Na_2SO_4, centrifuge, evaporate to dryness, dissolve in 1,2-dichloroethane	No clean-up		Premix	Menadione	173
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2 evaporate to dryness, dissolve in ACN	No clean-up		Animal feed	Menadione	180
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2–acetone (1:1), evaporate to dryness, dissolve in mobile phase	No clean-up		Infant formula	Phylloquinone	179
Preparative extraction using silica gel in N_2 atmosphere	Open-column SiO_2		Milk, colostrum	Vit. K	174
Homogenize in 2-propanol–hexane (66:34), centrifuge, evaporate hexane layer to dryness, dissolve in hexane, (1) Sep-Pak clean-up, evaporate to dryness, dissolve in hexane; (2) TLC, vitamin K band extracted with $CHCl_3$, evaporate to dryness, dissolve in ethanol	*(1) Sep-Pak* Silica	Hexane–diethyl ether (96:4)	Various meats	Vit. K_1, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, MK-10, MK-13	175–178
	(2) TLC Silica gel 60 F_{254}, 0.25 mm	LP–diethyl ether (85:15)			
Extract in water–methanol (60:40), centrifuge, MSB conversion to MD (5% Na_2CO_3 n-pentane), evaporate organic layer to dryness, dissolve in methanol	No clean-up		Animal feed	MSB	173

[1]. Open column and TLC are now sometimes applied as a clean-up procedure prior to HPLC injection. Hyphenated GC is preferably used for identification purposes.

3.4.1. Sample preparation

Owing to the high degradability of vitamin K, the samples cannot be submitted to alkaline saponification treatments [1,167]. Lipase hydrolysis for the extraction of PK and MK-5 from milk was chosen by Isshiki *et al.* [171] followed by a clean-up by preparative RPC, where vitamin K was monitored by UV detection (Table 12).

Mild alkaline hydrolysis of MSB extracted from animal feeds was carried out using ammonia solution [172] and Na_2CO_3 [173]. A preparative silica gel open-column method in a nitrogen atmosphere was used by Canfield *et al.* [174] to clean up the vitamin K extracted from human milk and colostrum.

K_1 and K_2 vitamers extracted from animal tissues [175] and vegetables [176] and K_3 vitamer from animal diets and food for livestock [177,178] were cleaned up in two steps: using a disposable silica column followed by a TLC on silica gel, collecting the vitamin K fraction by scraping off the band monitored by UV detection.

SFE extraction with CO_2 is a promising technique. Schneiderman and co-workers [179,180] developed a rapid, single-step sample preparation that allowed the quantitative extraction of PK from infant formula and of menadione from animal feeds within 15 min. No intermediate clean-up procedure was necessary, as the compounds trapped on silica gel were quickly eluted and then transferred to the HPLC mobile phase for direct injection.

3.4.2. High-performance liquid chromatography

NPC on a silica column was used only by Laffi *et al.* [172] for menadione determination, RPC mostly being applied [173,175–180] (Table 13). Two types of ODS columns were used by Isshiki *et al.* [171] for the separation of PK and MK-4 and for the MK vitamers, respectively. Canfield *et al.* [174] used a two-steps RP and two detectors.

The detection mode is the most interesting aspect of the development of newer HPLC methods. Three types of detectors have been reported. UV detection was used by Laffi *et al.* [172] for menadione determination and by Canfield *et al.* [174] in the first gradient step of HPLC analysis. This is a traditional detection mode [1]. The behaviour of the electrochemical reduction of 1,4-naphthoquinones was reviewed by Cadenas and Ernster [169], who pointed out the advantages of sensitivity and reliability of ED for K vitamers. Different types of ED were applied with either commercial or laboratory constructed detectors. The DL and the linearity were reported to be excellent: 2–5 ng for PK and 1–30 ng for MK-4 [171] and 125 ng for menadione [180]. The postcolumn formation of fluorescent reduced forms for FLD is a recent technique; the effluent from the analytical column is passed through a reduction cell and then reaches the detector in-line. Both chemical and electrochemical reduction were applied. Sakano *et al.* [176] for PK analysis in vegetables and Notsumoto *et al.* [177] for menadione in animal feed used $NaBH_4$ as the reducing agent, while Billedeau [173] used a column filled with zinc powder in-line between the HPLC column and the detector. Electrochemical reduction was used by Hirauchi *et al.* [175], coupling a commercial coulometric detector in line prior to the fluorescence detector. A simultaneous reduction and FLD of vitamin K_1 in a polyvitamin premix was investigated by Indyk [181]. The fluorescence was directly induced on the phylloquinone flowing through the flow cell of a recently developed commercial fluorescence detector which uses an intense (150 W) xenon source coupled with advanced electronics. This results in enhanced sensitivity (DL *ca.* 10 ng) which could help the development of FLD without postcolumn reduction devices.

3.4.3. Other chromatographic techniques

Haiduc *et al.* [182] determined vitamin K_3 in food supplements by GC on Chromosorb G with FID. They also used TLC on silica gel with a fluorescent indicator, followed by UV spectrophotometric detection at 267 nm of the extracted bands; they claimed a relative standard deviation of 5% in the range 4–40 μg/ml.

Coupled GC techniques (Table 14) have been applied to confirm the identification of the extracted vitamers from different matrices. Schneiderman *et al.* [180] identified menadione extracted from animal feeds by SFE by means of GC–MS. Billedeau [173] used GC–FT-IR for the identification of men-

TABLE 13

ANALYTICAL CONDITIONS FOR HPLC DETERMINATION OF VITAMIN K

For sample preparation see Table 12.

Stationary phase	Mobile phase	Detection	Analyte	Food	Ref.
For PK and MK-4: Partisil ODS 2 (5 μm). For MK-6–MK-9: Partisil ODS 3 (5 μm)	Methanol–ethanol–60% $HClO_4$ (60:40:0.12) containing 0.05 *M* $NaClO_4$; flow, 1 ml/min	ED, two GCE in series	PK, MK-4, MK-6, MK-7, MK-8, MK-9	Milk	171
LiChrosorb Si 60, 45°C	1,2-Dichloroethane; flow, 1.8 ml/min	UV, 251 nm	Menadione	Animal feeds	172
μBondapackC_{18}, 10 μm	ACN–0.25 *M* $NaClO_4$ (90:10)	ED (Ag electrode), −8.75V *vs.* SCE	Menadione	Animal feeds	180
μBondapack C_{18}, 10 μm	ACN–CH_2Cl_2–0.025 *M* $NaClO_4$ (90:5:5)	ED (Ag electrode), −1.1V *vs.* SCE	Phylloquinone	Infant formula	179
C_{18}, two-step elution	(1) Gradient elution; (2) isocratic elution, ethanol–hexane–water (90:6.5:3.5)	(1) UV; (2) ED	Vit. K	Milk colostrum	174
Nucleosil C_{18}, 5 μm	92.5% or 97.5% ethanol containing 0.25% $NaClO_4$; flow, 1 ml/min; post-column coulometric reduction	Fluorescence, 320/430 nm (ex./em.)	Vit. K_1, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9, MK-10, MK-13	Various meats	175
Nucleosil C_{18}, 5 μm	92.5% ethanol, postcolumn alkaline $NaBH_4$ reduction	Fluorescence, 320/430 nm (ex./em.)	(a) Vit. K_1, K_2; (b) menadione	(a) Various foods; (b) premix	(a) 176; (b) 177
Chemcosorb ODS-NH	45% Dioxane containing 0.2% $NaClO_3$, postcolumn coulometric reduction	Fluorescence, 320/430 nm (ex./em.)	MSB	Food from livestock	178
Supelcosil LC-18, 5 μm	Methanol–water (75:25), in-line postcolumn reduction with 20 × 2 mm I.D. column filled with Zn	Fluorescence, 325/425 nm (ex./em.)	MSB	Feeds	173

adione in feed extacts to confirm the results of a previously carried out HPLC method. The GC–FT-IR method gave a DL of 2 mg/kg *versus* 20 μg/kg in HPLC; therefore, it must be reserved for the qualitative analysis of menadione in synthetic feeds. GC coupled with automatic retrieval of spectra from computer libraries can be very useful in comparing analytical data and in evaluating the identification and purity of vitamin K extracts.

4. WATER-SOLUBLE VITAMINS

4.1. Thiamine (vitamin B_1)

4.1.1. High-performance liquid chromatography

The technique of extraction adopted (Table 15) is influenced by the mode of separation and detection selected. To detect thiamine and some of its derivatives, such as esters or coenzymes by using UV

TABLE 14

COUPLED GC METHODS FOR VITAMIN K IDENTIFICATION

Sample preparation	GC conditions	Detection	Analyte	Ref.
Extract in water–methanol (60:40), centrifuge, MSB conversion to MD (5% Na_2CO_3 in *n*-pentane), evaporate organic layer to dryness, dissolve in methanol	30 m × 0.32 mm I.D. DB-1701 column, 80°C (30 s), 16°C/min to 250°C (15 min); splitless injection, 200°C	FT-IR	MSB	173
SFE extraction, 15 min, CO_2, 8000 psi, 60°C, silica trap, elution with CH_2Cl_2, evaporate to dryness, dissolve in ACN	30 m × 0.25 mm I.D. DB-5 column, 150°C (2 min), 10°C/min to 250°C; splitless injection	Quadrupole MSD	Menadione	180

assay at 254 nm, a simple acid extraction followed by clean-up through disposable columns has been adopted. On the other hand, when the aim is to evaluate thiamine after derivatization to fluorescent thiochrome, an acid extraction with autoclaving, followed by enzymatic hydrolysis in the presence of diastatic and phosphorolytic enzymes and protein precipitation with trichloroacetic acid is used. The derivatization may be either pre- or postcolumn and consists of oxidation of the free thiamine with alkaline potassium hexacyanoferrate (III). This reaction is not stoichiometric, but it is reproducible under standard conditions.

Almost all the methods [19,183] employ RPC, using appropriate mixtures of methanol and either water or buffer solutions, with or without ion-pair modifiers. In most methods, however, the RP conditions chosen led to the co-elution of thiochrome monophosphate with thiochrome [184]. The DL depends on the method used: fluorimetric assay is more sensitive than UV assay. The latter technique could be suitable only for the determination of large amounts of thiamine (greater than 30 ng), because of the poor shape of the peak (broad with tailing), which further limits the sensitivity and increases the risk of interference from other components present in the extract [185]. The sensitivity and selectivity can be greatly increased by conversion to the fluorescent derivative thiochrome; in fact, FLD has a DL of 0.1 pmol [186]. On the other hand, Bertelsen *et al.* [184] found linear detector responses within the range 0.05–0.50 μg/ml of thiamine, with a DL of 0.5 μg per injection. They also reported that the relative standard deviation for the conversion of thiamine to thiochrome was 1.6%. Fernando and Murphy [187] proposed the neutralization of the precolumn-oxidized thiamine with concentrated H_3PO_4 in order to ensure a pH level acceptable to the C_{18} column and to eliminate possible pH-dependent alkaline degradation of thiochrome to its disulphide.

Nicolas and Pfender [188] described a simple sample preparation procedure without clean-up, which involves a two-step pH adjustment to deproteinize the sample followed by filtration, which provides a pure thiamine peak when the extract is chromatographed by IP-RP. Minor changes in the amount of IP reagent strongly affect the retention of thiamine, allowing the separation of the thiamine peak from potential interferents. Moreover, they also maximized the sensitivity of the method, minimizing the dilution during sample preparation, optimizing the UV detector response and using a large injection volume (100 μl).

4.1.2. Gas chromatography

Thiamine is a heat-sensitive and non-volatile compound, hence it cannot be directly determined by GC. Early attempts to prepare volatile derivatives of thiamine include the O-benzoyl, trimethylsilyl and trifluoroacetyl derivatives [189], but all these compounds have low volatilities at low temperatures and decompose at temperatures above 250°C. A group of indirect methods have therefore been developed, involving pretreatment with sulphite, which splits thiamine into the 5(2-hydroxy-

TABLE 15

CONDITIONS FOR HPLC DETERMINATION OF THIAMINE

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add 0.05 *M* H_2SO_4, autoclave for 30 min at 121°C, adjust pH to 4.6 with NaOH, dilute with water, filter through paper and 0.45-μm membrane	μBondapak C_{18}	Methanol 50 ml, acetic acid 5 ml, PIC B_5 12.5 ml, adjust to 1 l; flow, 1.2 ml/min	UV, 254 nm	IMF	191
According to AOAC [193] as modified by Ang and Moseley [194]. Conversion of thiamine to thiochrome	μBondapak C_{18} plus RP guard column	Water–methanol (70:30); flow, 1.5 ml/min	Fluorescence, 365/435 nm (ex./em.)	Chicken	184
Add 0.1 *M* HCl, heat for 30 min at 100°C, adjust pH to 4.5 with CH_3COONa, enzymatic digestion (β-amylase and takadiastase), filter through 0.2-μm cellulose acetate membrane. Conversion of thiamine to thiochrome. Purification on Sep-Pak	μBondapak C_{18}	Methanol–0.05 *M* CH_3COONa (pH 4.5) (60:40); flow, 1 ml/min	Fluorescence, 366/435 nm (ex./em.)	Dietetic foods	192
Add 5 *M* HCl, autoclave at 20 p.s.i. for 15 min, adjust pH to 4.5 with NaOH, centrifuge, filter through paper and 0.45-μm membrane. Thiamine conversion to thiochrome. Neutralization with H_3PO_4	Ultrasphere C_{18}	Acetonitrile–0.01 *M* acetate buffer (pH 5.5) (13:87); flow, 1.2 ml/min	Fluorescence, 366/435 nm (ex./em.)	Soybean, tofu	187
Dilute with water, adjust pH to 1.7–2.0 with 6 *M* HCl, add 5 *M* NaOH to pH > 4.0, adjust to volume with water, filter through paper	μBondapak C_{18} 50°C	0.15% Na hexane-sulphonate, 1.5% CH_3COOH, 0.1% EDTA, 20% methanol; flow, 2.5 ml/min	UV, 248 nm	Milk, infant formula	188

ethyl)-4-methylthiazole (HEMT) derivative. Among them, the method of Velísek and co-workers [189,190] involves acid and enzymatic (takadiastase) hydrolysis, cleavage by sulphite, extraction of the resulting HEMT with chloroform and direct GC analysis of the concentrated extract on a Carbowax 20M column at 220°C with the injection port and detector (FPD) temperatures set at 250°C. The sensitivity of the determination was about 0.1 μg/ml, comparable to thiochrome method results.

Velísek *et al.* [189] claimed some advantages of their GC method over HPLC and thiochrome methods: any gas chromatography equipped with FPD can be used; the clean-up procedures on base-exchanger silicates may be omitted; the method is applicable even in the presence of materials which either absorb thiamine or affect thiochrome fluorescence. On the other hand, an important drawback of this GC method is that the reaction cannot be performed in an "in-line" procedure, although this can be carried out for the HPLC analysis using the post-column thiochrome reaction.

4.2. Flavins (vitamin B_2)

Vitamin B_2 is a naturally fluorescent compound that occurs in foods in three principal forms: riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The chromatographic analysis provides a powerful tool for determining vitamin B_2 forms and for separating them from interfering substances, overcoming some of the drawbacks of the wet chemistry methods [196].

Early methods involved adsorption column chromatography, sometimes coupled with TLC, and fluorescence densitometric assay [197–199]. Nowa-

days, using HPLC we can determine simultaneously total riboflavin with other water-soluble vitamins [19,183].

4.2.1. High-performance liquid chromatography

Generally, HPLC methods for determining total RF are based on RPC both on C_{18} [200–203] and either on C_8 or on C_{18} in IP-RPC [187,204,205], although some procedures involving NPC have been proposed [206] (Table 16). Russell and Vanderslice [207] used polymer-based columns with an ACN–citrate–phosphate buffer gradient at 40°C in order to achieve the resolution of RF, FMN, FAD and 7-ethyl-8-methylriboflavin (I.S.). FLD is generally used, although some workers [202] applied UV detection. Even in this case, UV detection has lower sensitivity and specificity compared with FLD [207].

In several of the HPLC methods sample chromatograms suffer from poor resolution of the total RF peak, making its integration and quantification questionable.

TABLE 16

CONDITIONS FOR HPLC DETERMINATION OF FLAVINS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Add aq. CH_3COOH to pH 3, stir, centrifuge for 30 min, filter through 0.22-μm membrane	Cosmosil 5 C_{18}	Water–methanol–CH_3COOH (65:45:0.1); flow, 1 ml/min	UV, 254 nm	Milks	215
Grind to smooth paste, acid hydrolysis (0.125 *M* H_2SO_4, 121°C for 30 min), adjust pH to 4.6, enzymatic digestion (takadiastase, 25 min; papain, overnight), add TCA, heat for 5 min at 50–60°C, filter	Silica, 10 μm	Acetate buffer; flow, 1 ml/min	Fluorescence, 425/525 nm (ex./em.)	Blanched soya beans	206
Acid–enzymatic hydrolysis according to AOAC [196], filter through 0.45-μm membrane. For high-fat samples: extraction with hexane prior to filtration	LiChrosorb RP-8	5 m*M* hexanesulphonic acid–methanol (60:40); flow, 1 ml/min	Fluorescence, 440/565 nm (ex./em.)	Various foods	204
Degas under vacuum, filter	μBondapak C_{18}	Methanol–water (40/60); flow, 1 ml/min	Fluorescence, 450/525 nm (ex./em.)	Wines, champagnes	200
Add 0.1 *M* HCl, heat (100°C for 30 min), adjust pH to 4.5, enzymatic digestion (β-amylase and takadiastase), filter through 0.2-μm cellulose acetate membrane	μBondapak C_{18}	Methanol–0.05 *M* CH_3COONa (pH 4.5) (60:40); flow, 1 ml/min	Fluorescence, 422/522 nm (ex./em.)	Dietetic foods	192
Autoclave at 121°C for 15 min in HCl, adjust pH to 4–4.5, enzymatic digestion (takadiastase, 48°C, 3 h), filter through paper. Purification on Fluorisil and on C_{18} disposable columns	μBondapak C_{18}	Methanol–CH_3COOH–water (32:1:67), 5 m*M* Na hexanesulphonate	UV, 254 nm	Legumes, milk powders	195, 205
Homogenize in methanol and CH_2Cl_2 after addition of 7-ethyl-8-methylriboflavin (I.S.) Add 100 m*M* citrate–phosphate buffer (pH 5.5) containing 0.1% NaN_3, homogenize, centrifuge, filter	Act-I 80 A or 2 PLRP-S 100 A in series	Acetonitrile–10 m*M* citrate–phosphate buffer (pH 5.5) in gradient	Fluorescence, 360/550 nm (ex./em.)	Various foods	211

Generally, the method of extraction is similar to that for thiamine, *i.e.*, mineral acid/enzymatic extraction according to the AOAC official method [196], sometimes with some modifications [19,183]; this extraction method converts the protein-bound FMN and FAD into free forms and hydrolyses the phosphate forms to free RF, in order to measure the total vitamin B_2 activity. However, the hydrolysis step with takadiastase causes fluorescent interference in the sample extract, which could cause an overestimation of RF when FLD is used [187,205]. Hence a sample purification and concentration step using disposable cartridges, whose efficiency should be tested regularly and discharged if the amount of vitamin retained is less than 95% [208], is necessary.

To enhance the sensitivity of UV detection (DL 0.4 ng per injection), Vidal-Valverde and Reche [205] used a two-stage purification on Fluorisil and on a Sep-Pak C_{18} cartridge.

Hou and Wang [209,210] selectively detected RF and FAD utilizing a thin-layer amperometric detector with two electrodes in series. They found a DL of 40 ng for FAD and 4 ng for RF, and claimed that the dual-electrode detection offers superior selectivity over UV detection. Ollilainen *et al.* [203] compared the performances of FLD and UV detection and found a DL for RF of 20 pg per injection using FLD and 0.25 ng per injection using UV detection at 254 nm.

To determine simultaneously RF, FMN and FAD fluorimetrically in various foods, Russell and Vanderslice [211] developed a two-step non-degradative extraction procedure, using methylene chloride (to remove lipid artifacts and RF photodegradation products such as lumiflavin and lumichrome), methanol and 100 m*M* citrate–phosphate buffer (pH 5.5) containing 0.1% sodium azide (to maintain the pH within the range of flavin stability and to provide phosphate anion, as an inhibitor of phosphatase enzymes). They reported DLs of 0.21 ng for RF, 0.89 ng for FMN and 11.15 ng for FAD. To determine an RF content lower than 0.01 mg per 100 g, a trace enrichment technique could be used, by loading successive injections of the sample extract on to an RP guard column using water as mobile phase, the RF then being eluted as a sharp band by changing the mobile phase to methanol–water [208].

4.2.2. Other techniques

For qualitative studies, some advanced methods have been proposed recently. Asakawa *et al.* [212] developed an LC–MS system to overcome the problems imposed by the use of buffers as mobile phases. The RF peak is heart cut from the analytical column effluent, passed into sampling loops and adsorbed on a trapping column after dilution with the analytical mobile phase. Buffer constituents are washed out and the RF is eluted from the trapping column and re-chromatographed with a suitable mobile phase for its LC–frit-FAB-MS determination. The authors claimed that the system provides high sensitivity (300 ng per injection).

Kenndler *et al.* [213] developed a capillary zone electrophoretic (CZE) method to detect impurities (RF and RF mono- and diphosphates) in RF-5′-phosphate. They used a fused-silica separation capillary (100 cm × 100 μm I.D.), mounted in an HPLC–FLD system. This was modified by Kurosu *et al.* [214] in an immersed flow cell, improving the sensitivity of FLD in CE.

4.3. Niacin group

Niacin is found in foods as free acid (nicotinic acid) and the corresponding amide (nicotinamide); they exist as analogues in pyridine nucleotides (NAD and NADP). It is present also as nicotinoyl esters or NADH, which are not metabolically available. The determination of these compounds in foods is mainly carried out by using HPLC, although some workers prefer GC separations.

4.3.1. High-performance liquid chromatography

Depending on the form of the vitamin to be determined and the type of food, various methods of extraction have been developed (Table 17). Both acidic [216] and alkaline [217,218] extraction media have been used; however, it has been reported that the acidic extract could contain part of the niacin in bound form, which is therefore not available for the chromatographic analysis; in fact, HPLC values for the acid extract are consistently lower than those of alkaline extracts and of microbiological assay [208]. Therefore, when the purpose of the analysis is to determine the available niacin in foods, acid hydrolysis is preferred, as the alkaline procedure would release non-available niacin [219].

TABLE 17

CONDITIONS FOR HPLC DETERMINATION OF NIACIN

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Homogenize with ethanol, centrifuge, pass through Na_2SO_4 column, concentrate	LiChrosorb RP-18	Phosphate buffer–methanol–ACN–Bu_4NBr	UV, 260 nm	Meat	221
Homogenize in water, boil for 10 min, cool, filter through 0.45-μm membrane	Partisil SCX	50 m*M* phosphate buffer (pH 3)	UV, 260 nm	Meat	220
Add H_3PO_4, extract with methanol, homogenize, filter through paper and 0.45-μm membrane	FLC ODS, 50°C	1 mM Na dodecyl-sulphate–20 m*M* H_3PO_4–methanol (7:3) (pH 2.4)	UV, 261 nm	Meat, tuna	216
Extract in $Ca(OH)_2$, autoclave (15 min, 121°C), filter, adjust pH to 6.5 with oxalic acid, filter, clean-up on C_{18} column	LC-18-DB	Acetonitrile–0.1% H_3PO_4 and 0.1% Na dodecyl sulphate in water (23:77)	UV, 254 nm	Semolina, cheese, meat	218
Extract in 20% NaOH (100°C, 30 min), adjust pH to 7 with HCl, filter, clean-up on anion- and cation-exchange columns, filter through 0.45-μm membrane	Asahipak NH_2P-50	Acetonitrile–water (60:40) containing 0.075 *M* CH_3COONa	UV, 261 nm	Various foods	217
Acid hydrolysis (HCl, 121°C, 15 min), adjust pH to 4.5, enzymatic hydrolysis (takadiastase, 48°C, 3 h), filter through paper, clean-up on Dowex 1-X8	μBondapak C_{18} or Spherisorb ODS-2	Methanol–0.01 *M* CH_3COONa (pH 4.66) (1:9) containing 5 m*M* Bu_4NBr	UV, 254 nm	Legumes, meats	219

To determine both nicotinic acid and nicotinamide, a simple extraction with water [220] or with ethanol [221], followed by clean-up and/or filtration, is used. According to Vidal-Valverde and Reche [219], enzymatic hydrolysis after acid treatment is essential with legume samples, because of the high starch content of the hydrolysate. Further, the same workers stressed the need for the purification step prior to the chromatographic determination. They tested several purification methods and found that, using Dowex 1-X8 acetate resin conditioned with 1 *M* HCl, an optimum purification was achieved. The separation is generally carried out by IP-RP. As niacin has both acidic and basic groups, the IP reagent may be either an alkyl sulphate [216,218] or a quaternary ammonium salt [221].

However, some drawbacks have been reported: NP requires a long analysis time, whereas in the RP mode niacin moves with the solvent front or yields poor peaks, with tailing [222]. The IP-RP separation mode does not always ensure good retention time stability, and UV detection shows low sensitivity and selectivity in the presence of interfering compounds. To overcome these problems, Balschukat and Kress [223] proposed for feed products, a column-switching procedure in order to have an adequate separation of niacinamide from interferents; it performs the preparation of the niacinamide fraction on an RP column, followed by switching of the vitamin band on to a cation-exchange column for the determination, obtaining a complete chromatographic separation within about 30 min. The problems of low sensitivity and interferences could be overcome by using RP conditions with a gradient elution programme [208].

To improve the DL, a new approach, proposed for pharmaceutical products, could be the derivatization of niacin with N,N′-dicyclohexyl-O-7(methylcoumarin-4-yl)methylisourea (DCCI) to give a

highly fluorescent derivative; according to Finglas and Faulks [208], however, the use of this technique with food extracts could produce co-fluorescent compounds, which can require extensive sample clean-up and extract purification.

Using UV detection at 261 nm, Tsunoda *et al.* [216] found for both nicotinic acid and nicotinamide a DL of 0.1 mg per 100 g, while Balschukat and Kress [223] with UV detection at 264 nm coupled to a switching technique found for nicotinamide a DL of 0.5 ppm. A smaller amount of niacin (0.01 mg%) could be detected only when impurities arising from the hydrolysis process are removed by a proper clean-up procedure. For example, Hirayama and Maruyama [217] proposed a general procedure that can be used for various types of foods (high fat or protein contents, liquid or solids) consisting of alkaline hydrolysis, neutralization with acid, addition of the same volume of methanol to perform effective sample filtration for high-protein or carbohydrate-rich samples, elution from an AG 1-X8 anion-exchange column and additional clean-up on an IC-SP M cation-exchange column.

Tyler and Genzale [218] improved the UV detection limit to 0.004 mg per 100 ml by injecting niacin in a large volume (200 μl) of dilute H_3PO_4 which has a peak-sharpening effect and results in an increase in plate count approximate twenty times more than would be expected with an RP-18 phase.

4.3.2. Gas chromatography

The analytical approach differs according to the form of the vitamin to be determined (nicotinic acid or nicotinamide) (Table 18).

To detect nicotinamide, the simplest method involves its direct determination by GC with FID [224], but it lacks sensitivity. Another attempt involves the determination of nicotinamide as methyl nicotinate [225] by GC–FID, after ion-exchange separation, decomposition of nicotinamide and methylation in the presence of methanol and HCl. This method, however, is not specific and it is time consuming owing to the long time required for the methylation step.

Tanaka *et al.* [226] proposed a simpler procedure, which determines nicotinamide in meats and meat products after dehydration to 3-cyanopyridine with heptafluorobutyric anhydride. The product is over six times more sensitive than nicotinamide in GC and, after the reaction, no clean-up stage is necessary. They reported that the various possible interfering compounds present in the sample do not hinder the production and determination of 3-cyanopyridine. With this procedure they found a DL of 5 ppm and an average recovery of 98%.

To detect the total niacin content in various vegetables and animal products, Velísek *et al.* [227] esterified the free nicotinic acid with ethanol and after extraction, determined the ester by cGC on OV-1 using selective nitrogen–phosphorus detection (NPD).

4.4. B_6 vitamers

There are various biologically forms of vitamin B_6 usually present at very low levels in foods: pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), pyridoxal-5′-phosphate (PLP), pyridoxamine-5′-phosphate (PMP), pyridoxine-5′-phosphate (PNP) and the inactive pyridoxic acid (PA). Most of the methods proposed for the determination of vitamin B_6 have been based on HPLC, although other chromatographic techniques, such as GC [190] and MECC, have been used.

4.4.1. Sample preparation

Acidic media coupled with high temperatures (0.1 *M* H_2SO_4–HCl, 121°C) are required to denature proteins and to disintegrate the sample matrix, in order to promote extraction [19,183, 228–231]. However, these conditions cause the complete hydrolysis of the phosphate esters PNP, PLP and PMP to PN, PL and PM [232], respectively, and of PN-glucoside [233].

To determine all the vitamers individually, milder conditions, which do not cause dephosphorylation or deconjugation, such as an extraction medium containing a deproteinizing agent, has to be used [19,233]. The most commonly used deproteinizing compounds are perchloric acid [234], trichloroacetic acid (TCA) and sulphosalicylic acid (SSA). TCA can be removed from the extract by extraction with diethyl ether, while perchloric acid, which also extracts PA, can be precipitated by neutralization with KOH [19,233]. SSA is the most effective in deproteinizing samples and extracting B_6 vitamers, preventing enzymatic interconversion, but it is highly fluorescent so it must be removed by ion-

TABLE 18

CONDITIONS FOR GC DETERMINATION OF NIACIN

Compound	Detection	Column	Stationary phase	Solid support	Temperatures (°C)			Derivatization	Ref
					Injector	Column	Detector		
Nicotinic acid	NPD	25 m × 0.32 mm I.D. (silica)	OV-1 (0.25 μm)	–	230	50 (36 s), 20°C/min to 200	300	Esterification with ethanol	227
Nicotinamide	FID	2 m × 3 mm I.D. (glass)	5% OV-17	Chromosorb W AW DMCS (80–100 mesh)	190	130	190	Dehydration with heptafluorobutyric anhydride to 3-cyanopyridine	226

exchange chromatography prior to FLD [228,235]. This procedure leads to sample dilution, which makes the detection of low concentrations of B_6 vitamers difficult. To verify the presence of PLP and PMP, Bitsch and Möller [234] treated the samples with alkaline phosphatase (30 min, 25°C).

To determine all forms of vitamin B_6 in various food samples, Gregory and Sartain [232] evaluated the efficacy of both enzymatic and acid-catalysed hydrolyses of glucosylated forms of vitamin B_6. The enzymatic treatments were selective and included incubation with β-glucosidase, to measure PN-glycoside and other forms of vitamin B_6, and acid phosphatase treatment for the selective dephosphorylation of PLP and PMP. They found that the combination of these two enzymatic treatments would be useful in reducing the problem of interference in the measurement of PLP when the SSA extraction procedure is used.

TABLE 19

CONDITIONS FOR HPLC DETERMINATION OF B_6 VITAMERS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Extraction with MPA	Biosil ODS-5S	0.066 *M* KH_2PO_4 buffer, (pH 3); flow, 1 ml/min	Fluorescence, 290/395 nm (ex./em.)	Meats	229
Bound vitamers: autoclave (0.44 *M* HCl, 2 h), add SSA, stir, remove SSA with AG 1-X8 anion-exchange column. Non-bound vitamers: homogenize in 0.01 *M* CH_3COONa (pH 6.8), filter through paper and adjust pH to 4.7	RCM-100, 4 μm	(A) 0.033 *M* H_3PO_4, octane- and heptanesulphonic acid in water–2-propanol (97.5:2.5), pH 2.2. (B) 0.33 *M* H_3PO_4 in water–2-propanol (82.5:17.5), pH 2.2; flow, 1 ml/min	Fluorescence, 338/425 nm (ex./em.). Postcolumn reaction with $NaHSO_3$	Potatoes	228
Autoclave in 0.1 *M* H_2SO_4 at 121°C for 30 min, centrifuge	Novapak C_{18}, 5 μm, 35°C	Methanol–5 m*M* hexanesulphonic acid in 1% CH_3COOH (8:92); flow, 1.5 ml/min	Fluorescence, 290/390 nm (ex./em.)	Various foods	230
(1) All vitamers: homogenize in 0.1–0.5 *M* $HClO_4$, centrifuge, adjust pH to 7.5 with KOH, filter, adjust pH to 4 with HCl, filter through 0.45-μm membrane. (2) Esters: adjust pH to 4, enzymatic treatment (alkaline phosphatase, 30 min, 25°C), filter through 0.45 μm membrane	Lichrospher RP-18, 5 μm	(A) Methanol (B) 0.03 *M* phosphate buffer (pH 2.7) plus 4 m*M* octanesulphonic acid. Gradient of B from 90% to 69%, then to 90%	Fluorescence, 330/400 nm (ex./em.). Postcolumn reaction with $NaHSO_3$	Pork, liver, milk	234
Mix with 5% SSA, add I.S. solution (600 nmol/l 4-deoxypyridoxine), homogenize, add CH_2Cl_2, centrifuge, purify by anion-exchange chromatography, then either enzymatic hydrolysis (β-glucosidase, pH 5, 37°C, 2.5 h) or incubation in 6 *M* KOH (in the dark, 20°C, 3 h)	Ultrasphere ODS	(A) 0.033 *M* H_3PO_4–8 m*M* octanesulphonic acid (pH 2.2). (B) 0.033 *M* H_3PO_4–2-propanol (83:17). Gradient: 0 min, 17% B; 11 min, 31% B; 15 min, 100% B; held for 15 min; flow, 1 ml/min	Fluorescence, 295/405 nm (ex./em.)	Various foods	232
Add 0.5 *M* HCl, sonicate (10 min, 40°C), then autoclave (105°C, 10 min), add methanol, filter through paper, add TCA, (50°C, 10 min), centrifuge, adjust pH to 7, filter through 0.45-μm membrane	Novapak C_{18}	Methanol–5 m*M* hexanesulphonic acid in CH_3COOH (8:92); flow, 1.5 ml/min	Fluorescence, 290/395 nm (ex./em.)	Various foods	234

4.4.2. High-performance liquid chromatography

RP methods are of general use with various kinds of columns, eluting with isocratic mobile phases [19,183,236], while IP-RPC is used both isocratically [230,231] and with gradients [228,232,234] (Table 19).

Bitsch and Möller [234], employing IP-RPC with a binary gradient, achieved very good separations of all vitamers and PA in a relatively short time (about 30 min); the method was also highly reproducible.

PLP, the first B_6 vitamer eluted, can be separated from the solvent front more effectively using an Ultrasphere IP column than any other commercially available column [232]. However, to alleviate the problems encountered in the measurement of PLP in direct analysis, such as the presence of peaks eluted in the vicinity of PLP which render peak integration difficult or impossible, Gregory and Sartain [232] proposed an acid phosphatase treatment to hydrolyse PLP to PL.

Detection was mainly fluorimetric [229–232] and to enhance the fluorescence intensity some workers [228,234,235] carried out the $NaHSO_3$ postcolumn reaction of Coburn and Mahuren [237,238]. Bitsch and Möller [234] observed that the interferences due to matrix substances are minimized with this postcolumn reaction, because of the simultaneous shift from acidic to weakly alkaline pH.

Gregory and Sartain [232] and Bitsch and Möller [234] improved the accuracy and precision of their HPLC methods by using 4-deoxypyridoxine as a routine I.S., which is added at the time of extraction and behaves similarly to the naturally occurring B_6 vitamers during sample preparation. Under their analytical conditions this compound elutes between PN and PM, but Gregory and Sartain's [232] conditions provide longer retention times than those reported by Bitsch and Möller [234] with more resolved peaks.

Ang *et al.* [229] found with FLD of an MPA extract DLs of about 0.19, 0.1, 1.97 and 0.2 nmol for PMP, PM, PLP and PL, respectively, while Bitsch and Möller [234] claimed a DL ranging from 0.4 to 0.7 pmol for the seven vitamers, using postcolumn reaction with $NaHSO_3$.

Hou *et al.* [236] proposed ED for the determination of vitamin B_6 in multivitamin tablets, using a μBondapack C_{18} column eluted at 20°C with methanol–0.02 *M* phosphate buffer (pH 5) (10:90). They compared the use of UV detection at 254 nm, a glassy carbon electrode (GCE) and a carbon fibre microelectrode (CFE) with respect to linear range and DL. They pointed out that with the detector based on CFE, the working electrode can be reactivated only by chemical or electrochemical methods, and that after electrochemical pretreatment its sensitivity increases considerably. Generally, they found that the linear dynamic range for ED is wider than that for UV detection, with DLs (at a signal-to-noise ratio of 2) varying according to the vitamer and the detector. The DLs claimed were for PM, 1 ng, whatever the detection mode; for PL, 0.3, 1 and 0.5 ng with UV, CFE (1.2 V) and GCE (1.0 V), respectively; and for PN, 2.5 ng (UV and CFE) and 2 ng (GCE).

4.4.3. Micellar electrokinetic capillary chromatography

Swaile *et al.* [239] evaluated MECC for separating PLP, PA, PMP, PL, PM and PN and compared the results with those obtained by IP-RP. For MECC they used a 1 m × 75 μm I.D. fused-silica capillary column filled with 0.05 *M* SDS, 0.01 *M* Na_2HPO_4 and 0.006 *M* $Na_2B_4O_7$ in deionized water as mobile phase and an on-column laser-based FLD system equipped with a helium–cadmium laser ($\lambda_{ex.}$ 325 nm, $\lambda_{em.}$ 430 nm). For IP-RP the conditions were an Alltech C_{18} 10-μm column and a stepwise solvent gradient with 0.01 *M* sodium heptanesulphonate in 15% methanol in water (pH 2.75) as initial mobile phase, which after 12 min was changed to 45% methanol in water (pH 2.75) without an IP reagent.

They reported that the stepwise gradient separation of IP-RPC led to tailed peaks and baseline deviations when the mobile phase composition is changed. In addition, comparing IP-RPC and MECC, they found that, apart from the elution order being different for the two separations, the efficiency is far superior with MECC. They also pointed out that the composition of the mobile phase in MECC is critical and, to reduce the band-spreading effect, the surfactant concentration must be kept relatively high. They stated that capillaries of $\leqslant 50$ μm I.D. should be employed when laser-based FLD is possible; otherwise, larger diameters (50–100 μm I.D.) are necessary when absorbance detection is used.

Yik *et al.* [240] studied a system for interfacing ED with MECC which couples the separation capillary column to the detection capillary, with a section of porous graphite tubing which forms an electrically conductive joint. They pointed out that the graphite joint has to be kept immersed in a buffer reservoir, to prevent it from drying out, together with the ground electrode of the high-power source. The reservoir is electrically insulated from the electrochemical cell containing the carbon fibre electrode. They claimed that their ED system allows better sensitivity than laser-excited FLD, with a DL two orders of magnitude lower than those reported for laser FLD (0.4 fmol), while the linear dynamic range of the calibration plot was slightly over two orders of magnitude (from *ca.* 1 to 200 ppm).

4.5. Folacin

The folacin complex consists of a group of related compounds derived from pteroylglutamic acid, exhibiting varying physiological activity, bioavailability and stability. It includes folic acid (FA), 7,8-dihydrofolic acid (DHF), 5,6,7,8-tetrahydrofolic acid (H_4folic acid), 5-methyltetrahydrofolic acid (5-CH_3-H_4folic acid), 5-formyltetrahydrofolic acid (5-CHO-H_4folic acid) and 10-formyltetrahydrofolic acid (10-CHO-H_4folic acid) plus *p*-aminobenzoylglutamic acid (PBGA) and pterine-6-carboxylic acid (PT-6-COOH). In most plant and animal materials, they exist in long-chain polyglutamyl forms, with chain lengths ranging from five to seven γ-linked glutamyl residues [24].

4.5.1. Sample preparation

The major problems during extraction and clean-up are the lability of folacin both to oxidation and thermal treatment [19] and the conjugation of polyglutamyl forms of the vitamin. An additional complication is the extremely low level present in most foods, less than 100 ng/g [242] (Table 20).

The thermal degradation kinetics of 5-CH_3-H_4folic acid in both the presence and the absence of oxygen were studied by Barrett and Lund [243]. To protect 5-CH_3-H_4folic acid from degradation during the thermal extraction procedure, Schulz *et al.* [244] developed a method for removing oxygen from food samples by ultrasonication followed by flushing with nitrogen and by the addition of sodium ascorbate as an antioxidant. This technique of extraction was also applied by Bitsch *et al.* [245] to determine folates from cabbage. They achieved the autolysis of polyglutamates using a raw cabbage extract as a source of endogenous conjugase activity.

Engelhardt and Gregory [246] evaluated the properties of pteroylpolyglutamate hydrolase (conjugase) from hog kidney and the efficacy of this enzyme in deconjugating sample extracts for folate analysis. They stated that the appropriate combination of enzyme concentration and incubation time should be determined for each type of sample, as extracts of a variety of foods caused detectable inhibition of the enzyme.

The addition of antioxidants (ascorbate and 2-mercaptoethanol) to sodium phosphate buffer (pH 4.5) was adopted by Holt *et al.* [247] for milk and dairy products, whereas lyophilization followed by incubation in trifluoroacetic acid was used by Andondonskaja-Renz and Zeitler [248] for royal jellies and caviar.

For citrus juices, White [249] carried out a solid-phase extraction procedure using a phenyl-bonded phase conditioned with tetrabutylammonium phosphate, which permitted the separation of 5-CH_3-H_4folic acid from sample interferences. The folate was then easily eluted from the cartridge with a buffer–methanol mixture without IP reagent. He found that ascorbic acid was the main interferent, and hindered the selective detection of 5-CH_3-H_4folic acid when amperometric detection was used. After solid-phase extraction the ascorbate concentration was considerably reduced, facilitating the detection of the analyte. This purification procedure was automated by White *et al.* [250], using a switching ten-port valve that allowed direct injection of filtered juice into the HPLC system. Sample clean-up was effected on a C_{18} precolumn followed, after the elution of ascorbic acid (monitored by UV detection), by the backflushing of 5-CH_3-H_4folic acid to the analytical column. This procedure was claimed to be effective in reducing oxidative losses of folate during sample preparation.

4.5.2. High-performance liquid chromatography

RP separation methods with buffer–organic mobile phases is still preferred [248–251], although IP methods are also used [19,247]. Hahn *et al.* [251]

tested several chromatographic systems based on IP-RPC and conventional RPC. They found that the best resolution of folacins is achieved by using a 3-μm ODS Hypersil column in a gradient of 5 m*M* KH_2PO_4 (pH 2.3) and ACN.

UV detection is considered sufficient for normal contents of folates [245], whereas for small amounts FLD is necessary [251]. Because of the low specificity of UV detection, Bitsch *et al.* [245] used LC–MS to identify the various forms of folate in cabbage. Hahn *et al.* [251] studied the postcolumn derivatization of folacins, paying particular attention to the influence of the flow-rate of the derivatization reagent (1% $K_2S_2O_8$) on the fluorescence of the resulting products, finding that higher flow-rates resulted in smaller peak areas.

They also compared their DLs with literature data, finding that, with UV detection at 295 nm, the values were lower than those in the literature (DHF 1.77, H_4folic acid 2.67, 5-CH_3-H_4folic acid 1.15, 5-CHO-H_4folic acid 2.37 ng per injection). The same was found for fluorimetry (365/450 nm for DHF and H_4folic acid and 295/356 nm for 5-CH_3-H_4folic acid and 5-CHO-H_4folic acid), the DLs being 0.89, 0.04, 0.007 and 0.28 ng per injection for DHF, H_4folic acid, 5-CH_3-H_4folic acid and 5-CHO-H_4folic acid, respectively. However, fluorimetry does not achieve the sensitivity of ED [251].

4.6. Vitamin C

Vitamin C occurs in two biologically active forms: *l*-ascorbic acid (AA) and *l*-dehydroascorbic acid (DHAA), its oxidized form. Nowadays the most common mode of analysis is HPLC, which overcomes the main drawbacks of chemical methods in the presence of interfering compounds in the matrix of foods, as it is more selective and sensitive.

4.6.1. High-performance liquid chromatography

There is a wide range of HPLC methods [19,183]; the most common modes of separation are RP, IP-RP and weak anion-exchange with an NH_2-bonded phase, with various columns, elution conditions, detection systems and extraction techniques (Table 21). Most methods, however, suffer from an AA retention time that is too close to the void volume, which may lead to errors in quantitative work.

The type of extraction media and stabilizing solutions in order to prevent AA oxidation are very important. A well established extractant and stabilizer is metaphosphoric acid (MPA) [252–260]. Gennaro and Bertolo [253] stressed that the addition of MPA prevents the decay of ascorbic acid during the first few hours, losing its efficacy after about 12 h. In fact, according to Bushway *et al.* [257], vitamin C begins to degrade within 15 min after homogeniza-

TABLE 20

CONDITIONS FOR HPLC DETERMINATION OF FOLACIN VITAMERS

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Homogenize, adjust pH to 4.5 with CH_3COOH, centrifuge, add sodium phosphate buffer (pH 4.5) (10% ascorbate, 1 *M* 2-mercaptoethanol), incubate with conjugase, centrifuge, filter through 0.45-μm membrane	Microsorb C_{18}, 3 μm	Methanol–phosphate buffer (pH 6.8) and Bu_4NBr; flow, 1 ml/min	Fluorescence, 238/340 nm (ex./em.). Postcolumn oxidation with hypochlorite	Milk dairy products	247
Lyophilize, suspend in 0.1 *M* trifluoroacetic acid, stir at 37°C for 20 min, centrifuge, adjust pH to 4 with 2 *M* K_3PO_4	Spherisorb ODS, 5 μm	3.5 m*M* K_2HPO_4 (pH 6.5); flow, 1 ml/min	Fluorescence, 360/460 nm (ex./em.)	Royal jelly, caviar	248
Centrifuge (2°C, 15 min), adjust pH to 5 with NaOH, (a) direct solid-phase extraction, (b) conjugase hydrolysis (37°C, 1 h) and solid-phase extraction	Zorbax ODS	Methanol–acetate buffer (pH 5.5) (25:75); flow, 1 ml/min	ED, +200 mV *vs.* Ag/AgCl, 3 *M* NaCl DAD	Citrus juices	249

TABLE 21

CONDITIONS FOR HPLC DETERMINATION OF ASCORBIC ACID

Sample preparation	Stationary phase	Mobile phase	Detection	Food	Ref.
Degas by filtration through paper, dilute 1:20 with pH 9 buffer prior to injection	HPICE-AS1	Acetonitrile–10 m*M* H_2SO_4 (4:96); flow, 0.8 ml/min	PAD, +0.70 V	Beer	278
Add 62.5 m*M* MPA, blend, centrifuge, filter through paper	Aminex HPX-87H, pore size 9 μm	4.5 m*M* H_2SO_4; flow, 0.5 ml/min	DAD, 245 nm	Potatoes, strawberry	252
Extract in 0.5% oxalic acid, add perchloric acid, centrifuge, two-step derivatization with 4-ethoxy- and 4-methoxy-phenylenediamine, purify through C_{18} and Aminex 50W-X2 (Na^+) columns	PRP-1	16% ACN in 50 m*M* H_3PO_4 and 5 m*M* propanesulphonate, adjust pH to 9 with 0.15 *M* H_3PO_4; flow, 1 ml/min	Fluorescence, 375/475 (ex./em.)	Dairy products	273
Filter through Nucleopore Syrfil, 25 mm, 0.45-μm filter and dilute 1:5	LiChrospher RP-8, 5 μm	Octylammonium-salicylate, 5 m*M*; flow, 1.0 ml/min	UV, 254 nm	Milks	262
Homogenize in MPA, ultracentrifuge and filter through Nucleophore Syrfil, 25 mm, 0.45-μm filter	LiChrospher RP-18,5 μm	Octylammonium-salicylate, 5 m*M*; flow, 1.0 ml/min	UV, 254 nm	Fruit juices	253
Blend in 2.5% MPA–mobile phase (1:2), filter through paper, clean-up through Sep-Pak C_{18} and 0.45-μm nylon 66 membrane. To detect total AA: reduce DHAA with DTT	Rainin NH_2, 8 μm	ACN–0.05 *M* KH_2PO_4 (75:25); flow, 2 ml/min	UV, 254 nm	Apple, potatoes	254
Homogenize, add 0.1 *M* citric acid containing 5 m*M* EDTA together with hexane, centrifuge, filter through 0.45-μm membrane.	Three Ultrasphere ODS C_{18} in series, 4°C	Buffer (pH 5)–0.1 *M* NaH_2PO_4–5 m*M* EDTA–5 m*M* tetrabutylammonium phosphate	Fluorescence, 350/430 nm (ex./em.) after postcolumn derivatization	Luncheon meats	261
Homogenize in 5% MPA, centrifuge, filter through paper, dilute, clean-up through Sep-Pak C_{18} and filter through 0.45-μm membrane	μBondapak NH_2	5 m*M* KH_2PO_4 (pH 4.6)–ACN (30:70); flow, 1 ml/min	UV, 254 nm	Vegetables	256
Mix with ethanol and MPA, centrifuge. For total AA: add 0.3 *M* Na_3PO_4 and NaSH (20 min, 35°C), dilute with MPA, filter through 0.45-μm membrane. For AA: dilute with MPA and filter through 0.45-μm membrane	Cosmosil 5 C_{18}, 40°C	MPA (2 g/l); flow, 1 ml/min	UV, 243 nm	Citrus juices	266
Homogenize in 0.2 *M* phosphate buffer (pH 2), extract with 3% MPA, filter through 0.45 μm nylon filter	PLRP-S, 5 μm 100 Å	1.8% H_4folic acid and 0.3% MPA in water; flow, 0.5 ml/min	DAD, 244 nm	Fruit, vegetables, juices	257
Add 12.5% TCA solution to precipitate proteins, centrifuge, filter. For total AA add homocysteine to reduce DHAA to AA, adjust pH to 7.0 (15 min, room temp.)	Nucleosil 7 C_{18}	2 m*M* Bu_4NOH in water (pH 2.92); flow, 1.5 ml/min	UV, 254 nm	Milk	264

corrected 17 June 93/AP

In third column, second row from the bottom:

"1.8 % H_4 folic acid" should read "1.8 % THF"

tion and by 30 min 4–5% is oxidized. When large subsamples are used, they suggested replacing the expensive MPA with a phosphate buffer (pH 2) in the initial homogenization step. However, the extraction medium can cause problems during the chromatographic separation. For example, Graham and Annette [252], investigating the use of amino-bonded and RP-18 columns with and without IP to determine AA extracted using MPA, found that amino-bonded phases were unsatisfactory in analysing cooked samples because of a poor resolution and a co-eluting component contaminating the AA peak; likewise, RPC with or without the use of IPC leads to column poisoning. Vanderslice and Higgs [255], studying a large variety of fresh and cooked vegetables, fruits, fruit juices and cereals, also found that the MPA–acetic acid extraction was more efficient than the citric acid extraction used previously [261]. However, MPA extraction led to irreproducible separations on ODS and this was replaced with a microporous polystyrene–divinylbenzene polymer (PLPR-S) column, which gave clean and stable traces even when MPA extraction was used.

Gennaro *et al.* [262] compared the performances of various types of RP packings (C_{18} and C_8, spherical and irregular, with 5- and 10-μm particles and different degrees of end-capping), with salicylates of heptylamine, octylamine and decylamine as the ion interaction reagent. They found that a longer alkyl chain of the amine causes higher retention but, at the same time, the longest alkyl chain shows the lowest sensitivity. Further, greater retentions corresponded to stationary phases characterized by spherical packings and by the highest carbon loading. In fact, these parameters affect the degree of functionalization induced on the column by the interaction reagent [262]. They concluded that the most suitable reagent was octylammonium salicylate, which with UV detection permits a DL of the order of 113 pmol injected [253]. Gennaro *et al.* [263] proposed an RP ion interaction reagent method which makes use of chiral compounds as the interaction reagents, to separate D-(−)- and L-(+)-AA. After having employed the optical isomeric forms of malic, tartaric and mandelic acids as the interaction reagents, they achieved a good separation of AA using a Spherisorb ODS-2 column with 0.005 *M* octylamine D-(−)-tartrate.

Sapers *et al.* [254] developed a method to determine ascorbic acid 2-phosphate (AAP), without phosphatase treatment, simultaneously with AA using an aminopropyl column with an isocratic separation which ensures no interfering peaks. The retention times of AAP (isocratic, 30 min) could be reduced by gradient elution, increasing the proportion of buffer to 50%. DHAA was determined after reduction to AA with dithiothreitol (DTT). To determine AA, DHAA, isoascorbic acid (IAA) and dehydroisoascorbic acid (DHIAA) simultaneously, Vanderslice and Higgs [261] used three Ultrasphere ODS columns at 4°C in series and an eluting buffer (pH 5) of 0.1 *M* NaH_2PO_4, 5 m*M* EDTA and 5 m*M* tetrabutylammonium phosphate. After separation on the column, the compounds were converted into fluorescent derivatives by a method involving oxidation of AA and IAA with mercury(II) chloride to DHAA and DHIAA and subsequent reaction with *o*-phenylenediamine. This procedure allows determination at the 3-ng level.

Most methods determine DHAA as the difference between total AA after DHAA reduction and the AA content of the original sample [252] in order to determine total AA with UV detection. The sensitivity of DHAA is insufficient to detect the amounts usually found in foods even when it is monitored at its maximum wavelength (210 nm). The reduction of DHAA to AA can be accomplished using homocysteine [252,259,264] or dithiothreitol solution [254,265], and usually TCA or MPA is added to the sample prior to the reduction treatment to prevent AA and DHAA from being oxidized to diketogulonic acid (DKG) and other degradation products. Sawamura *et al.* [266] reduced DHAA to AA with sodium hydrosulphide and claimed that NaSH has advantages over DTT and homocysteine.

On the other hand, Graham and Annette [252] claimed that using DAD at 230 nm, AA and DHAA could be detected simultaneously if the concentration of DHAA is sufficiently high, as its DL is 100 ng/μl. Otherwise, DHAA must be determined only after reduction to AA. With their procedure (a modification of the homocysteine method), they found almost complete reduction of DHAA to AA (96%) and a DL for AA of 1 ng/μl.

However, UV detection at a single wavelength is the most often used method [253,254,256,259,260, 262,265.267–270].

Some workers detected AA with fluorescence detection after precolumn [271] or postcolumn derivatization of DHAA. Huang and Kissinger [272] proposed a highly selective method to determine AA and DHAA simultaneously using UV and ED in series. DHAA was derivatized postcolumn with *o*-phenylenediamine and the product was detected at 348 nm, while AA was detected at a glassy carbon electrode maintained at +600 mV *vs.* Ag/AgCl. Bilic (273] set up a procedure for the simultaneous determination of AA and DHAA in dairy products using a two-step precolumn derivatization with 4-methoxy- and 4-ethoxy-1,2-phenylenediamine; the derivatives formed, presumably methoxy- and ethoxyquinoxalines, exhibit a tenfold increase in fluorescence compared with the quinoxaline derived from 1,2-phenylenediamine. First, the sample is derivatized with 4-ethoxy-1,2-phenylenediamine, then the sample is derivatized with 4-ethoxy-1,2-phenylenediamine, then the derivatives are isolated and retained by a RP adsorbent. The AA is oxidized to DHAA with saturated aqueous bromide and then derivatized with 4-methoxy-1,2-phenylenediamine and retained on the same RP adsorbent. The recovery of derivatives from the RP adsorbent is accomplished by elution with 15% ACN and 0.5% trifluoroacetic acid in water. It was claimed that, at a signal-to-noise ratio of 3, the DLs were about 50 and 70 fmol of AA and DHAA, respectively, per 5-μl injection.

ED has been used either alone [274,275] or in series with UV detection [272,276,277]. To detect AA and sulphite in beers, Wagner and McGarrity [278] found that the use of a pulsed amperometric detector with a single applied voltage was unsuccessful, as the loss of detector sensitivity was extremely rapid, probably owing to the contamination of the surface area of the platinum electrode used. As an alternative, they obtained good results utilizing a standard amperometric cell, like Kitada *et al.* [258], and superior results were achieved when it was operated in a pulsed mode with cleaning cycles continuously applied during the analysis. Kitada *et al.* [258] found a DL of 5 μg/g using an amperometric detector set at +500 mV *vs.* Ag/AgCl.

Bode and Rose [279] studied the detection of AA based on coulometric ED, evaluating the time necessary for the complete reduction of DHAA to AA by β-mercaptoethanol, the optimum pH of the reduction reaction and the necessary ratio of reductant to DHAA. A high degree of sensitivity was achieved with detection of less than 1 pmol of AA.

4.6.2. Other techniques

Usually, AA is evaluated by TLC after reaction with dinitrophenylhydrazine [280]. The ascorbic acid osazone derivative can be purified by column chromatography [280] or measured by TLC and densitometry [281], achieving a DL of 5 ng, or detected *in situ* at 494 nm [282] with a DL of 1 ppm. To determine total AA, Touchstone *et al.* [281] oxidized AA to DHAA with copper(II) salts prior to reaction with dinitrophenylhydrazine. To separate AA from its oxidation products (DHAA and DKG), Tsuda and Fukuba [283] used Avicel SF as adsorbent, ethyl acetate–pyridine–water (10:4:3) as eluent and 2,6-dichlorophenolindophenol sodium salt in ethanol as spray reagent.

Application of GC to the determination of vitamin C has been on a limited scale, and only a few papers have dealt with food applications [190]. As AA is a non-volatile compound, it can be determined after derivatization as the trimethylsilyl ether on non-polar methyl polysiloxanes as stationary phase with FID. The same derivatization procedure could be suitable for obtaining volatile trimethylsilyl derivatives from DHAA and DKG, which therefore could be simultaneously determined with AA. During the derivatization, basic solvents such as pyridine have to be avoided, owing to the instability in alkaline media of AA and its oxidation products.

AA may be extracted from food using MPA, and after clean-up on a cellulose column the AA in the dried eluate is derivatized and determined [284]. An interesting approach could be the use of isotachophoresis to identify AA and its oxidized products in foods. As only acidic media have to be selected for the leading electrolyte, β-alanine is more suitable as a counter ion, as it has a wide buffer action at pH 3–4; *n*-caproic acid, having the lowest solubility, is better as the terminal solution [283]. To detect AA in wheat dough, Kvasnicka *et al.* [285] carried out a capillary isotachophoresis with a 200 × 0.8 mm I.D. preliminary column and a 200 × 0.3 mm I.D. separation column. As initial electrolyte they used 5–10 m*M* HCl, 12–24 m*M* β-alanine and 0.1% hydroxyethylcellulose, while the final electrolyte contained 5 m*M* caproic acid and 5 m*M* Tris buffer.

Similarly, Tsuda and Fukuba [283] found that the optimum conditions for the isotachophoretic determination of AA were HCl–β-alanine at pH 3.6 and *n*-caproic acid at pH 3.4 as the leading and terminal solutions, respectively.

With UV detection of AA at 254 nm, Kvasnicka *et al.* [285] found a DL of AA of 0.1 mg/kg. Using the same system, they detected DHAA after its oxidation to AA with 2-mercaptoethanol. In fact, DHAA does not appear in the isotachopherogram, even if it is present in the sample. To confirm this, Tsuda and Fukuba [283] determined the oxidation products of AA by both TLC and isotachophoresis. TLC confirmed the presence of DHAA, although in the isotachopherogram only AA and DKG were found.

5. CONCLUSIONS

This survey of the recent literature confirms that there is a trend towards the use of HPLC methods as standard techniques for the qualitative and quantitative determination of both water- and fat-soluble vitamins. To date, the single chromatographic determinations of some vitamins, such as cyanocobalamin, pantothenic acid, biotin and some pseudovitamins, have not been reported for food products, probably because nutritional scientists are not particularly interested in these vitamins; hence there has not been further research on the assay methods [19,183].

Most HPLC methods use bonded phases, particularly RP packing materials; smaller particles (3 μm), shorter columns and microbore columns are increasingly being used to improve speed and sensitivity. Although UV and FLD are widely employed, ED is becoming increasingly important for the detection of very small amounts of vitamins, as it shows enhanced sensitivity and selectivity.

The use of HPLC column-switching techniques makes the sample preparation less laborious and, at the same time, prevents some oxidizable vitamins from being oxidized. These automatic procedures are becoming more important for routine work in food control laboratories, where speed of analysis and simple sample preparation together with a reliable and reproducible chromatographic assay are needed.

Chromatographic techniques other than HPLC are seldom employed in food analysis and they are reserved for research purposes and not used as routine analytical methods.

For identification purposes, DAD, MS and FT-IR could be advantageous detection techniques, especially for the development of reference methods to validate HPLC determinations of vitamins.

6. ABBREVIATIONS

AA	Ascorbid acid
AAP	Ascorbic acid 2-phosphate
ACN	Acetonitrile
AOAC	Association of Official Analytical Chemists
CE	Capillary electrophoresis
CFE	Carbon fibre electrode
cGC	Capillary gas chromatography
CN	Cyano, nitrile
cZE	Capillary zone electrophoresis
DAD	Diode-array detection
DHAA	1-Dehydroascorbic acid
DHF	7,8-dihydrofolic acid
DHIAA	Dehydroisoascorbic acid
DKG	Diketogulonic acid
DL	Detection limit
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECD	Electron-capture detection
ECN	Effective carbon number
ED	Electrochemical detection
FA	Folic acid
FAB	Fast atom bombardment
FAD	Flavin adenin dinucleotide
FID	Flame ionization detection
FLD	Fluorescence detection
FMN	Flavin mononucleotide
FPD	Flame photometric detection
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GCE	Glassy carbon electrode
GPC	Gel permeation chromatography
H_4folid acid	5,6,7,8 Tetrahydrofolic acid
HPLC	High-performance liquid chromatography
IAA	Isoascorbic acid

I.D.	Inside diameter
IMF	Intermediate moisture food
IP-RPC	Ion-pair reversed-phase chromatography
I.S.	Internal standard
LC	Liquid chromatography
LP	Light petroleum
MD	Menadione
MECC	Micellar electrokinetic capillary chromatography
MeOH	Methanol
MK	Menaquinone
MPA	Metaphosphoric acid
MS	Mass spectrometry
MSB	Menadione hydrogensulphite
MSD	Mass spectrometric detector
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NARP	Non-aqueous reversed-phase
NP	Normal phase
NPC	Normal-phase chromatography
NPD	Nitrogen–phosphorus detection
ODS	Octadecylsilica
PA	Pyridoxic acid
PAD	Pulsed amperometric detection
PBGA	*p*-aminobenzoyl glutamic acid
PK	Phylloquinone
PL	Pyridoxal
PLP	Pyridoxal-5′-phosphate
PM	Pyridoxamine
PMP	Pyridoxamine-5′-phosphate
PN	Pyridoxine
PNP	Pyridoxine-5′-phosphate
Pt-6-COOH	Pterine-6-carboxylic acid
RF	Riboflavin
RP	Reversed-phase
RPC	Reversed-phase chromatography
RP-FC	Reversed-phase flash column
SCE	Saturated calomel electrode
SDS	Sodium dodecyl sulphate
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SSA	Sulphosalicylic acid
TCA	Trichloroacetic acid
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TOC	Tocopherol
UV–VIS	Ultraviolet–visible
UV	Ultraviolet

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Review

Chromatographic analysis of antibiotic materials in food

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ABSTRACT

The monitoring of food materials for antibiotic residues is an area of increasing concern and importance due to the potential impact on human health. Large-scale screening applications require methods that are rapid, accurate, provide low detection limits and are free from interference. The problem is further complicated by the wide range of chemical functionalities and modes of operation exhibited by the antibiotic materials of physiological significance in use today. As demonstrated, chromatographic methods provide many of the advantages necessary for screening applications. Judicious choice of sample preparation method, separation mode and detection strategy can provide significant immunity from problems associated with the food matrix.

Gas chromatography can provide extremely high separation efficiencies, however, only a limited number of antibiotic compounds are inherently volatile enough for direct analysis by gas chromatography. Derivatization to enhance the volatility of the antibiotic is one approach to overcome this limitation. Among the methods available, reversed-phase high-performance liquid chromatography is used extensively for the analysis of many antibiotic systems as it does not require derivatization and it combines relatively high separation efficiencies with low detection limits. The diverse group of properties exhibited by the antibiotic materials in use today suggests that the choice of detection strategy is a key component in the successful development of an analysis technique. Derivatization of the antibiotic material is frequently used to add either a fluorogenic of chromogenic moiety to the antibiotic compound to enhance detection. Derivatization procedures suffer from several limitations which are problematic when making measurements in complicated food matrices. Among the different detection modes ultilized for antibiotic analysis, polarimetric detection has the potential to provide extremely selective detection of most antibiotic materials, and this selective response can minimize many of the constraints placed upon the separation system by the sample matrix. Although many of the separation modes used for antibiotic analysis are well developed, separations based on capillary electrophoretic methods have much potential in the field of antibiotic analysis. Future investigations are needed to extend the generality of these techniques and expand their use into the field of food analysis.

CONTENTS

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1. INTRODUCTION

Antibiotics are an extremely important class of compounds as they represent a key component in the strategy used to control bacterial infections in both human and animals. Chemically, the collection of materials which exhibit antibiotic properties are a diverse group with widely divergent chemical functionalities and modes of operation. This diversity, though, presents a tremendous challenge to the analyst as subtle structural variations in closely related antibiotic materials can lead to pronounced differences in the chemical toxicity and biological activity of the antibiotic. Thus there is the need to develop rapid analytical methods for antibiotic materials which could be used to screen suspect products for their presence.

Traditional microbiological assays, that is, those methods that involve the growth of a probe microorganism on a medium containing the antibiotic, suffer from a variety of limitations including the time and labor-intensive nature of the procedure. In addition, microbiological assays cannot differentiate among the various forms and derivatives of a given antibiotic system, and the quantitative information offered by such an approach reflects the total amount of all forms of a given antibiotic, rather than providing distinct quantitative information on different analogues. This information can be very useful as it will be shown that the relative distribution of various analogues in a given antibiotic system is unique and can be indicative of the source of the particular antibiotic sample. Thus, knowledge of the distribution of components can be useful from both a physiological perspective, that is, one analogue usually has minimal toxicity and maximum antibiotic activity, and for recognizing the source of contamination.

In contrast to microbiological methods, chromatographic approaches can provide a rapid response and offer both high sensitivity and separation efficiencies. Thus, chromatographic methods have the potential to provide many of the characteristics necessary for systematic screening of food materials. However, the extremely diverse nature of antibiotic materials requires that a variety of different separation modes, detection strategies and sample preparation procedures be used to achieve the goals outlined previously as necessary for rapid and sensitive screening. Several recent reviews have appeared that describe various chromatographic strategies for the separation of antibiotic materials found in food materials [1–3]. These reviews are limited in their scope and application as they key either on a specific antibiotic system or on only a few antibiotics. Ref. 1 provides an excellent summary of extraction/deproteinization systems used for isolating a variety of antibiotic materials found in different food matrices.

This work will focus on chromatographic methods for separating antibiotic compounds found in food materials. Methods selected for inclusion have demonstrated enhanced selectivity towards the various structural analogues in a given system, and, in many cases, the high separation power of the chromatographic system minimizes the amount of sample preparation required. Novel detection strategies will also be discussed which can impart additional selectivity to the method. Finally, problems inherent to antibiotic analysis in specific food matrices will be presented with the goal to highlight those aspects of the separation system that are necessary to minimize, or eliminate, interferences from the matrix.

2. SEPARATION MODES FOR ANTIBIOTIC ISOLATION AND ANALYSIS

2.1. Gas chromatography

Gas chromatography (GC) possesses the requisite chromatographic resolution to separate closely related antibiotic analogues. However, most antibiotic materials of physiological significance are not sufficiently volatile for direct GC analysis. In situations where the antibiotic material is present at extremely low levels, the high sensitivity of the electron-capture or thermionic detector may be advantageous and justify the time necessary to derivatize the antibiotic to enhance its volatility. For example, chloramphenicol is a highly regulated antibiotic which may be present in food materials at extremely low levels and which can severely impact human health in susceptible individuals at these levels. Allen [2] has reviewed eight different GC methods in which chloramphenicol is derivatized using either trimethylsilane (TMS), hexafluorobutylacetate (HFBA) or ethyl acetate [4]. Electron-capture detection provides a limit of detection (LOD) at the 1–10 ng/g level in most food matrices with recoveries ranging from 72% (unpolished rice) to >90% in milk and meat. In general the added steps necessary for derivatization are problematic and methods based on liquid chromatography (LC) have received much recent interest.

2.2. Thin-layer chromatography

Thin-layer chromatographic (TLC) methods can provide high sample throughput which is advantageous in screening applications. In general, the method has been limited in the past by poor chromatographic resolution, minimal selectivity and high detection limits. Oka and co-workers [5,6] have demonstrated that high-performance TLC (HPTLC) can solve several of these problems. As is evident from Fig. 1, six of the main tetracycline analogues can be separated by HPTLC with excellent chromatographic resolution. Reversed-phase TLC (RP-TLC) (C_8) was used to alter the elution order to aid in identification of the different analogues

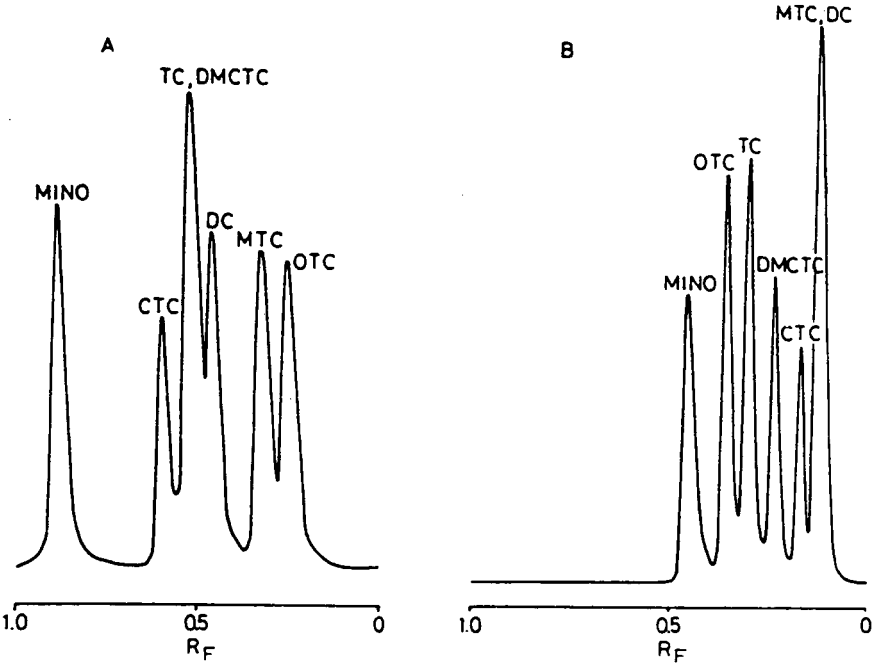

Fig. 1. Elution profiles for selected tetracyclines separated by HPTLC. (A) Pre-developed silica gel HPTLC plate saturated with Na_2EDTA and activated for 2 h at 130°C. Solvent system: chloroform–methanol–5% Na_2EDTA (65:20:15). (B) RP-TLC plates. Solvent system: methanol–acetonitrile–0.5 *M* oxalic acid (1:1:4), pH 3.0. From ref. 5.

and minimize possible interferences from the food matrix. Detection requires either the addition of a visualization agent (Fast Violet B Salt) to the plate with heating to create a colored product, or direct ultraviolet (UV) densitometry can be used. Visualization agents provide a rapid response but are only semi-quantitative in their response.

2.3. Liquid chromatography

2.3.1. Reversed-phase chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) combines rapid sample throughput, high sensitivity (detection mode-dependent) and high chromatographic selectivity. However, the expense of efficient HPLC columns and the ease with which they can be damaged suggest that it would be prudent to pretreat food extracts to limit irreversible adsorption of highly hydrophobic materials on the column. For example, both sulfonamides [7] and tetracyclines [8] have been extracted from animal tissues under acidic conditions and the extractants cleaned up by the use of a Sep-Pak C_{18} cartridge. Sulfonamides were eluted from the cartridge with methanol, while the tetracyclines were eluted using a mixture of dimethylformaldehyde (DMF)–water (4:6). The eluted antibiotic mixtures were then separated into their respective structural analogues by RP-HPLC. With the Sep-Pak treatment the recovery of the sulfonamides ranged from 77 to 103%, however, tretracycline recovery was less than 60% for most animal tissues.

The separation power of HPLC with respect to the differentiation of closely related structural analogues of antibiotic materials is evident in Fig. 2 [9]. In Fig. 2, RP-HPLC is used to separate ampicillin and the (5*R*,6*R*)- and (5*S*,6*R*)-epimers of penicilloic acid. These epimeric forms of penicilloic acid have identical molecular masses but differ solely by the arrangement of atoms at one chiral center in the molecule. Near-baseline resolution is obtained for the epimeric pair allowing accurate and precise quantitation of each of the compounds.

In situations where more detailed structural analysis of the separated antibiotics is required for precise identification, HPLC separation has been combined with mass spectrometric (MS) detection. However, the vacuum requirements of the mass

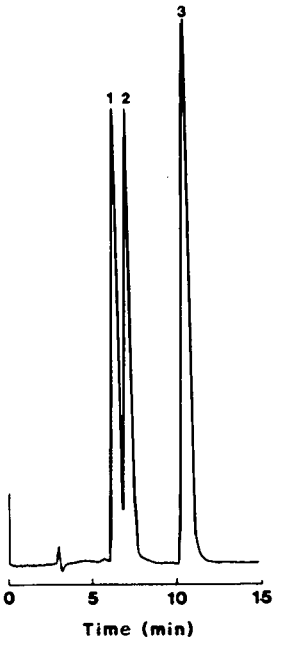

Fig. 2. Separation of (5*S*,6*R*)- (1) and (5*R*,6*R*)- (2) epimers of penicilloic acid and ampicillin (ABPC) (3). Concentrations of the 5*S*,6*R*- and 5*R*,6*R*-epimers were approximately 10 μg/ml, that of ABPC 12.5 μg/ml. Injection volume: 20 μl. Eluent: 20 m*M* phosphate buffer (pH 4.2)–methanol (3:1, v/v) at a flow-rate of 0.8 ml/min. Absorption detection (290 nm) at a sensitivity of 0.04 a.u.f.s. Reaction coil length was 2 m. From ref. 9.

spectrometer are incompatible with the solvent throughput characteristic of conventional HPLC columns. Moore *et al.* [10] have solved this problem by using 150 mm × 0.3 mm I.D. microbore columns. The analytical column was preceded by a 30 mm × 0.3 mm I.D. guard column. The use of the microbore column reduced the mobile phase flow-rate by a factor of 200 which allowed direct connection of the column to the mass spectrometer. The authors avoided mobile phases containing salts which easily crystallize (*e.g.* phosphate) to minimize column blockage. The combined HPLC–MS system provided efficient detection and identification of cephalosporins at the 1–5 ng level.

2.3.2. Ion-pair chromatography

Many antibiotic systems possess functional groups which are ionized at pH values compatible with silica-based RP-HPLC stationary phases. Therefore, the formation of an ion pair with the

antibiotic material can produce very efficient separations with additional modes of chromatographic control. Mobile phase pH (which controls the extent of ionization), the type of counter ion (which governs the interaction with the stationary phase and formation constant of the ion pair) and the concentration of the ion-pair reagent (to drive the equilibrium) can all be varied to control the separation. Yoneda *et al.* [11] has used 0.2 *M* HCl as an eluent to separate aminoglycoside antibiotics. This aggressive mobile phase did not inhibit the post-column detection reaction with *o*-phthalaldehyde and recoveries from beef were of the order of 80% with detection limits at the 0.2-μg/g level. Voydsner *et al.* [12] utilized a variety of alkylsulfonates to achieve efficient separation of several penicillin derivatives extracted from bovine milk and separated on a 2.1 mm I.D. column. Both C_8 and C_{12} alkylsulfonates were used, either separately or in combination. The choice of both ion-pair reagent and concentration was found to be useful in enhancing the separation efficiency and in minimizing interference from co-extracted components of the milk. The alkylsulfonates did not affect the detection mode as both UV–photodiode array (PDA) and thermospray MS were used. The UV–PDA system gave slightly better detection limits, but the mass spectrometer produced both $[M-H]^+$ and $[M-Na]^+$ ions which provided unambiguous identification of each penicillin analogue.

2.3.3. Immunoaffinity chromatography

An innovative approach to minimize the matrix effect of samples such as eggs and milk has been demonstrated by Van de Water *et al.* [13]. An antibody-mediated clean-up routine has been used to provide excellent recovery of chloramphenicol from these samples. In this approach, a monoclonal antibody to chloramphenicol was immobilized on a carbonyldiimidazole-activated support. The high specificity of the interaction allowed large samples to be processed and concentrated. Conventional HPLC was used to access residual matrix effects after the immunoaffinity column clean-up. Fig. 3 shows the results of this study. The immunoaffinity column is extremely selective and, as is evident, no extraneous peaks were observed within the elution window for chloramphenicol that would interfere with quantitation. This was true for both the egg and milk samples. The column was very durable when the antibiotic was eluted with a mixture of glycine (0.2 *M*) and NaCl (0.5 *M*, pH 2.8) and a single column was reused over 30 times. However, it was also found that recoveries with the immunoaffinity column were lower than those obtained using a conventional solid-phase extraction procedure. One possibility is that the chloramphenicol is protein-bound and either lost in the precipitation step, or the protein complex cannot be bound by the immunoaffinity column. Further study is necessary to understand and control this limitation.

2.4. Supercritical fluid chromatography

Packed-column supercritical fluid chromatography (SFC) with UV detection has been evaluated as a separation technique for the analysis of sulfonamides found in swine kidney extracts [14]. Theoretically, SFC maintains much of the separation power available in capillary column GC without requiring that analytes be volatile and thermally stable. The mobile phase consisted of supercritical carbon dioxide with varying concentrations of methanol as a modifier. Other modifiers were tested (*e.g.* DMF) but were found to be of limited use in modifying the chromatographic properties of the mobile phase. Both silica and amino-bonded stationary phase columns were used and each demonstrated distinctly different selectivities with respect to the family of sulfonamides. It was also observed that mobile phase modification with methanol provided different effects with the two columns in that the amino-bonded phase was much more sensitive to modifier variations. The authors thus suggest that the differing selectivities observed for the different stationary phases and modifier concentrations can be used to choose a specific separation system for a specific problem.

2.5. Capillary electrophoresis

Capillary electrophoretic (CE) methods utilize a small-diameter silica capillary (25–75 μm) as the separation vessel with applied field strengths that approach 500 V/cm to separate ionic materials. Much recent work has focused on the high separation efficiencies possible with this separation mode and 10^6 plates/m have been demonstrated. Ideally,

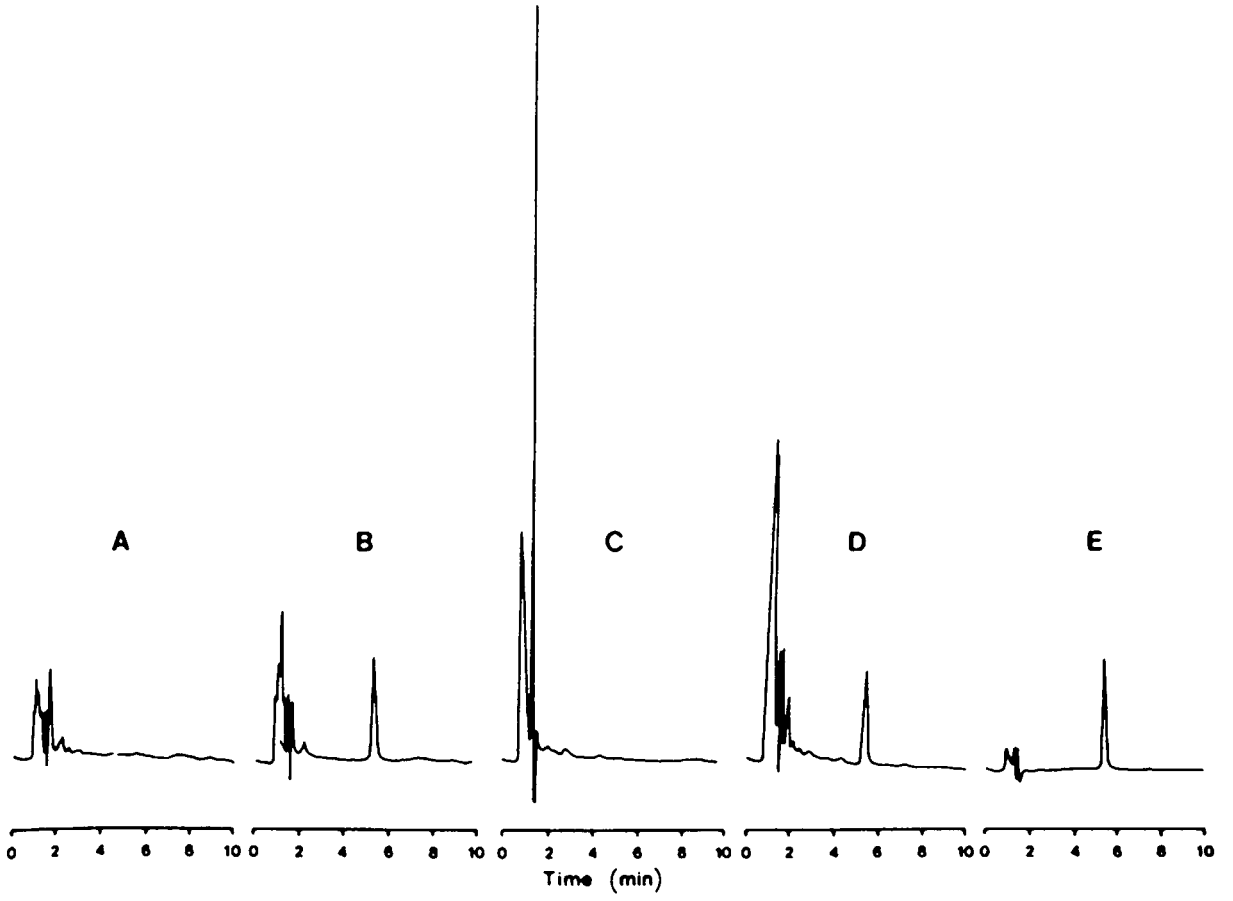

Fig. 3. HPLC analysis of chloramphenicol (CAP) obtained from milk and egg samples and purified by means of antibody-mediated clean-up. (A) Blank milk sample; (B) spiked (10 μg/kg) milk sample; (C) blank egg sample; (D) spiked (10 μg/kg) egg sample; (E) standard solution of CAP. Absorbance detection at 0.016 a.u.f.s. From ref. 13.

these characteristics make CE particularly well suited for the separation and analysis of antibiotic materials found in food matrices. However, many antibiotic compounds are neutral, or only minimally ionized over large pH ranges, and therefore not directly amenable to the conventional CE technique.

Nishi and co-workers [15,16] utilized micellar electrokinetic chromatography (MEKC) to separate both penicillin and cephalosporin antibiotics. In this mode, sodium dodecyl sulfate (SDS), a surfactant, is added to the electrophoretic buffer at a level above the critical micelle concentration (CMC). The micelle phase provides a new avenue of selectivity as the neutral antibiotics can differentially partition into the interior of the micelle. Migration in MEKC for these antibiotic materials was found to depend on the inherent electrophoretic mobility of the analyte, the distribution of the analyte in the micellar phase and the ability of the analyte to ion pair with the micelle. In this mode, anionic, cationic and neutral or zwitterionic materials can be separated in a single system. Fig. 4 shows a CE and MEKC separation of nine cephalosporin antibiotics. The high separation efficiency available with this technique is clearly evident in the three electropherograms. The extension from the CE to the MEKC mode (A to B) shows the enhanced selectivity available with the micelle phase. C shows

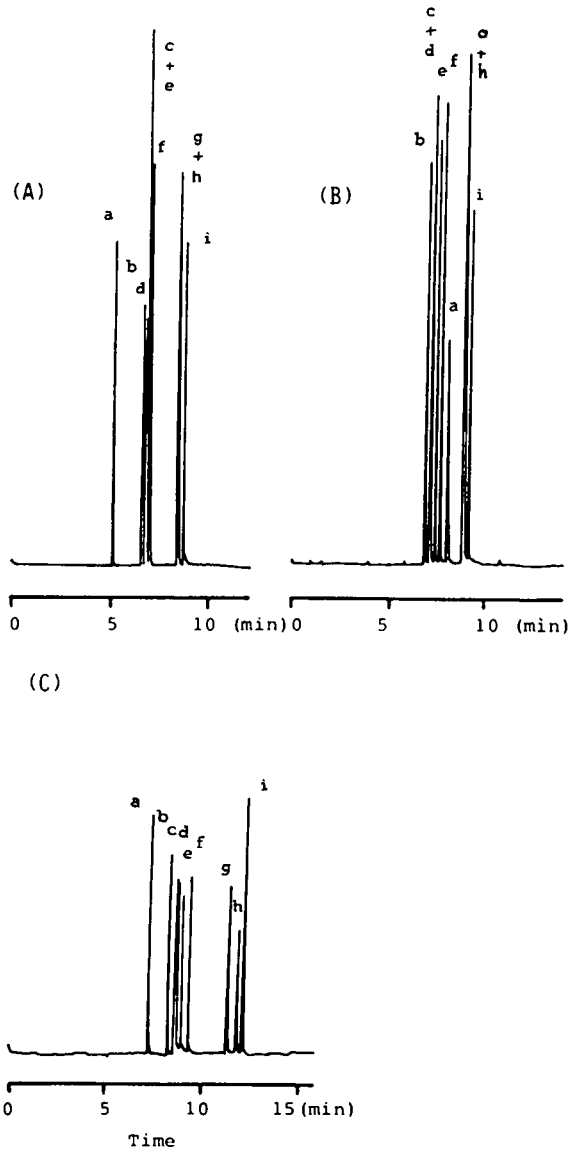

Fig. 4. Effect of tetraalkylammonium salts (TAA) on the micellar electrokinetic chromatographic (MEKC) separation of nine cephalosporin antibiotics: (a) C-TA; (b) ceftazidime; (c) cefotaxime; (d) cefmenoxime; (e) cefoperazone; (f) cefpiramide; (g) cefpimizole; (h) cefminox; (i) ceftriazone; (A) CZE mode with 0.02 *M* phosphate–borate buffer (pH 9.0); (B) MEKC mode with 0.02 *M* phosphate–borate buffer (pH 9.0) containing 0.05 *M* SDS (sodium dodecyl sulfate); (C) MEKC mode with 0.04 *M* TMAB (tetramethylammonium bromide) added to the same SDS solution as in (B). Conditions: buffer, 0.05 *M* acetate buffer; applied voltage, 20 kV; temperature, ambient; detection wavelength, 210 nm. From ref. 16.

the dramatic effect the addition of tetraalkylammonium (TAA) salts to the CE buffer can have as all nine antibiotics are separated in a single run. It was suggested that the TAA salts form ion pairs with anionic materials, and the partitioning of the neutral ion pair into the micelle is increased thereby increasing the retention of the solute.

Another approach is to use capillary isotachophoretic chromatography to separate penicillin and cephalosporin antibiotics and their precursers [17]. Judicious choice of the leading buffer system can be used to separate the various analogues of a given antibiotic. In general, these reports demonstrate the large potential CE has with respect to the analysis of antibiotic materials in food. High separation efficiencies and multiple separation modes and mechanisms provide a number of different strategies which can be used to analyze antibiotics in complicated matrices. It is clear that this potential will drive the technology into new areas and applications which have proven difficult to solve by conventional chromatographic approaches. The present limitation to the application of this technology is the need to provide more reproducible sample introduction and detection techniques.

3. DETECTION MODES TO ENHANCE SELECTIVITY AND SENSITIVITY

3.1. General overview

The diverse group of properties exhibited by the pharmacologically significant antibiotics makes both their separation and detection difficult. Several antibiotic systems possess inherent properties which facilitate detection. Chloramphenicol, for example, exhibits strong absorption above 260 nm as a consequence of its aromatic structure. However, in most cases, alternative strategies have to be applied in order to enhance detection sensitivity due to the low residual levels at which many of these materials must be monitored. The problem is further compounded by the fact that a detection strategy that works for one antibiotic system may not work for another. Yet in many treatment regimes, two or more antibiotics may be used simultaneously to extend the range of susceptible organisms. There have been several reports of detection systems which are universal in their response. However, in the case of

MS, cost, sensitivity and limitations due to co-eluting materials have precluded its adoption for routine applications. As will be discussed, polarimetric detection is a possible detection strategy which is almost universal in its response to antibiotic materials.

Very few antibiotic systems possess chromophores which are accessible above 230 nm. Alternatively, one can use short-wavelength UV detection, however, in this spectral region, many materials absorb appreciably and chromatographic mobile phases must be chosen with respect to both their chromatographic and spectroscopic properties. For example, Terada and Sakabe [18] combined ion-pair chromatography with UV detection at 210 nm to separate and analyze penicillins found in milk. A Sep-Pak clean-up procedure proceeded the chromatographic analysis. However, even with this procedure, many materials were detected during the elution window of the three penicillins tested. In order to differentiate the antibiotic materials from other components of the milk sample, penicillinase was added to degrade the penicillins. Subsequent chromatographic analysis of the degraded sample and comparison with the original chromatogram showed the absence of three peaks which corresponded to the penicillins. In general, for routine screening of widely diverse samples, short-wavelength UV detection is not sufficiently selective. The complicated nature of the chromatographic data obtained in this mode will make low-level identification and quantitation difficult, or impossible.

3.2. Derivatization to enhance detection

Chemical transformation of an antibiotic substance through the addition of either a chromophore or a fluorophore can be used as a detection strategy to enhance the materials detectability. This approach adds time, complexity and cost constraints to the procedure. For pre-column derivatization, the chromatographic resolution of closely related substances can be jeopardized as the added moiety may dominate the chromatographic properties of the derivative. Thus subtle variations in the chromatographic properties within a group of related analogues may be lost through the derivatization procedure. Further, derivative stability will be a main consideration when choosing the appropriate analytical procedures to use in a given situation. Post-column derivatization does not directly affect the chromatographic properties of the antibiotic, however, the reaction chemistry must be rapid on the chromatographic time scale in order to preserve the chromatographic information. Further, the post-column reaction chemistry may be affected by the chromatographic conditions and this could limit the types of mobile phases that may be used.

Pre-column derivatization procedures have been developed for a number of antibiotic systems and several representative examples are summarized in Table 1. In general, chemical derivatization becomes more difficult to implement as the complexity of the sample matrix increases.

TABLE 1

SELECTED PRE-COLUMN DERIVATIZATION AGENTS FOR ANTIBIOTIC MATERIALS

Antibiotic	Derivatizing agent	Detection mode	Ref.
Erythromycin	Disodium-2-(stilbyl-4″)-4-(naphthol-1′,2′:4,5)-1,2,3-triazole 2″-6′-disulfonate	Absorbance	19
	Ethylsuccinyl chloride	Fluorescence	20
Penicillins	4-Bromethyl-7-methoxycoumarin	Fluorescence	21
Gentamicin	2,4-Dinitrophenyl derivatives	Absorbance	22
	1-Fluoro-2,4-dinitrobenzenen	Absorbance	23
β-Lactams	Imidazole–metal salt	Absorbance	24
Mononsin	9-Anthryldiazomethane	Fluorescence	25
Inophores	9-Anthryldiazomethane	Fluorescence	26
Sulfonamides	Fluorescamine	Fluorescence	27
Fortimicin A	3,5-Dinitrobenzoyl Chloride	Absorbance	28

3.3. Polarimetric detection

Most antibiotic materials and their derivatives possess the property of chirality, that is, they are optically active. This is a consequence of the fact that they are produced by living organisms which use chirality as part of the system of molecular recognition. Therefore, optical activity is an extremely rare characteristic and it is usually associated with biological activity, past or present. The application of optical activity detection to the analysis of antibiotic materials should provide many of the advantages characteristic of the ideal detection system. Assuming that the sensitivity of the optical activity detection system is sufficient to handle the extremely dilute conditions encountered in HPLC, the detector would provide significant specificity without requiring derivatization prior to detection. Thus the separation system could be chosen with regard only to the separation problem.

Several recent reports have described the application of optical activity detection to the study of antibiotic materials after separation by HPLC. The laser-based polarimeter used for these studies utilizes a helium–neon laser (He–Ne) to achieve rotational detectabilities at the 5–10 μdegree level [29]. The instrumentation uses relatively unsophisticated components, and commercial polarimetric detection systems are now available.

Specific rotation, $[\alpha]$, is the physical parameter used to describe the innate optical activity of a substance. Since optical activity is such a rare property, specific rotation measurements on eluting materials could have potential in identifying closely related structural analogues in a given antibiotic system.

Laser-based polarimetric detection has been applied to the study of erythromycin in milk [30]. Several significant results were inferred from this work. First, as expected, polarimetric detection provided a selective response which removed some of the constraints placed upon the separation system by the complicated milk matrix. The milk sample was extracted with a 4:1 mixture of pentanol and chloroform and the extractant injected directly into the HPLC system. The polarimetric detection system provided significant specificity as only a few peaks, in addition to those due to erythromycin, were observed. Refractive index detection of the same solution showed that there were many more components eluting from the HPLC column. Further, the polarimetric system was very sensitive providing minimum detectable quantities at the 10-ng level for erythromycin. Finally, and most importantly, specific rotation measurements were made on the antibiotic materials as they eluted from the HPLC column. Commercial erythromycin preparations consist of three closely related analogues designated as erythromycins A, B and C. The specific rotation obtained for erythromycins A and C differed by almost 10% even though the structural variation which differentiates these two analogues consists of the substitution of a hydroxyl (C) for a methoxy (A) group. This difference entails a mass difference of approximately 2%. Thus, specific rotation measurements have potential in identifying closely related structural analogues when studying their fate or location in complex physiological pathways or biological matrices.

The sensitivity of specific rotation to subtle structural variations was tested in the analysis of the epimers of ticarcillin and carbenicillin [31], two members of the penicillin family of antibiotics. Each epimer contains a total of four chiral centers, but they differ by the arrangement of atoms at a single one of these centers. Polarimetric detection of these epimers after separation by HPLC provided detection at the 10-ng level. More importantly, specific rotations were obtained for each epimer as it eluted from the separation system. The results of these measurements are summarized in Table 2 below. The large difference in specific rotation measured for the epimeric forms of these two materials is very surprising given the minor structural variation which differentiates the two forms. Clearly, polarimetric detection of antibiotic materials has demonstrated significant advantages from both a qualitative and a quantitative perspective when compared to other detection methods used for these materials.

As seen in the erythromycin study discussed previously, organisms which produce antibiotic substances frequently produce several closely related structural analogues. The relative abundance of these different analogues can vary depending upon the conditions used during the growth phase of the organism. Thus, the relative distribution of the different analogues in a given system can be indicative of the source of that antibiotic sample and this information may be useful for identification.

TABLE 2

SPECIFIC ROTATION MEASUREMENTS FOR SIX PENICILLIN ANALOGUES

Compound	Specific rotation (deg $dm^{-1}g^{-1}$ ml)		
	Literature	Direct measurement[a,b]	Calculated from peak height[a,b]
Penicillin G	310	340 ± 11	347 ± 6
Ampicillin	281	314 ± 11	321 ± 6
(10*R*)-Carbenicillin	–	192 ± 8[c]	180 ± 1
(10*S*)-Carbenicillin	–		213 ± 1
(10*R*)-Ticarcillin	–	179 ± 8[c]	166 ± 1
(10*S*)-Ticarcillin	–		210 ± 2

[a] 632.8 nm.
[b] Experimentally determined standard deviations.
[c] Epimeric mixture.

This hypothesis was recently tested for gentamicin in which reversed-phase ion-pair chromatography, in combination with laser-based polarimetric detection, was used to analyze milk samples containing this antibiotic materials [32]. A representative chromatogram for gentamicin samples obtained from two different sources is given in Fig. 5. Gentamicin does not possess a chromophore which is accessible above 230 nm. Therefore, previous studies have utilized chemical derivatization of the gentamicin to render it either fluorescent or absorbing. However, the derivatized gentamicin analogues were difficult to separate as the subtle differences which distinguish the four analogues were lost and under optimum conditions only three of the four main analogues were resolved. The polarimetric detection system does not require derivatization prior to detection, and separation of the four main components was now possible. Finally, with all four components resolved, it is evident that the two gentamicin samples contain different amounts of the four analogues and this information may be of use in identifying the source of the sample.

This hypothesis was tested using a milk sample spiked with gentamicin. The chromatogram presented in Fig. 6 shows the reversed-phase ion-pair

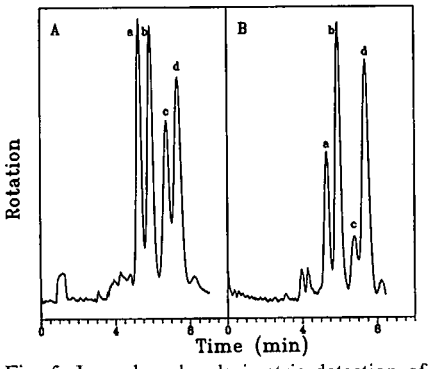

Fig. 5. Laser-based polarimetric detection of gentamicin analogues from two commercial gentamicin preparations after separation by reversed-phase ion-pair chromatography. (A) Supplier 1; (B) supplier 2. Total amount injected was 10 μg. Elution order: a, C_{1a}; b, C_2; c, C_{2a}; d, C_1. Eluent: methanol–0.4 *M* TFA in water (20:80, v/v); flow-rate: 0.75 ml/min. The disturbance in chromatogram A at approximately 1 min is the standard signal produced by a DC Faraday coil corresponding to a rotation of $2.86 \cdot 10^{-4}$ deg. From ref. 32.

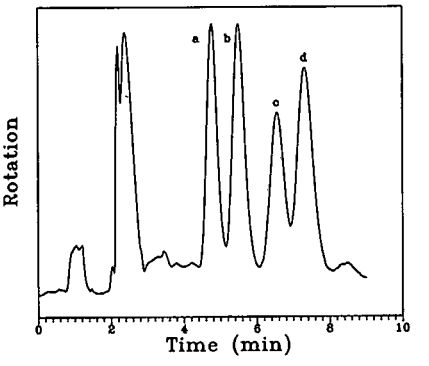

Fig. 6. Separation and detection of gentamicin analogues extracted from milk. Experimental conditions same as in Fig. 5. From ref. 32.

separation of gentamicin analogues obtained from a milk sample. The selectivity of the polarimetric system is a distinct advantage when working with complicated food matrices. Each milk sample was acidified with trifluoroacetic acid (TFA), centrifuged for 10–15 min, and the supernatant liquid was then injected directly into the HPLC column without further preparation. Thus, including the chromatographic run, less than 25 min per sample are required for analysis. Comparison of Figs. 5 and 6 clearly shows the source of the gentamicin sample used to spike the milk.

Polarimetric detection, in combination with HPLC separation, has demonstrated significant advantages for the analysis of optically active antibiotic compounds found in food materials. The approach is fairly universal for most pharmacologically important antibiotics and sensitive detection can be achieved without the need to derivatize the antibiotic. Non-derivatized materials are easier to resolve chromatographically. The polarimetric detection system can provide information on the innate optical activity of eluting antibiotics and this information cannot be obtained by any other means. Specific rotations can be used to identify closely related structural analogues even when present in a complicated food matrix.

3.4. Mass spectrometry

MS is another detection mode that can theoretically provide detection for all antibiotic substances. Two limitations preclude widespread utilization of this detection mode for screening applications; the cost and complexity of the instrumentation, particularly LC–MS, and the difficulty in obtaining diagnostic molecular ions which can be used for both identification and quantitation. A variety of different ionization modes have therefore been used to provide reproducible ion formation. In general, the mass spectral information is usually used for qualitative purposes, while a separate UV detector is then used in-line in order to provide enhanced quantitative information.

Sulfonamides have been determined in salmon flesh using ion-spray tandem MS (MS–MS) [33]. The MS–MS technique, using collision-induced dissociation, provided unique structural information which was used to distinguish between isomeric and isobaric sulfonamides. The MS information was very useful for identification purposes, however, UV PDA detection was used to provide a limit of detection at the 25-ng/g level.

Thermospray (TSP) MS was used to study gentamicin analogues separated by reversed-phase ion pair HPLC using TFA as the ion-pair reagent [34]. Fragmentation patterns were used to identify the four main components. Several previous studies utilized alkylsulfonates as ion-pair reagents for gentamicin separation. However, it was found that these salts were not compatible with the TSP source and TFA was therefore used. Detection limits for the HPLC–TSP-MS technique were approximately an order of magnitude higher than those reported using fluorescent derivatization.

Particle beam HPLC–MS has been evaluated as a potential method for the analysis of antibiotic compounds found in food materials [35]. The sensitivity of the particle beam interface was found to be related to the heat capacity of the solvent system with the highest sensitivity observed for solvents with the lowest heat capacities (*i.e.* methanol > acetonitrile > water). This would limit to some extent the range of mobile phase compositions available for use with this detection system. A LOD at the ng/g level was demonstrated under single-ion monitoring conditions. The particle beam system was also compared to the TSP source. For the same antibacterial compounds, the TSP source provided less structural information than the particle beam, however, the LOD for the particle beam was approximately a factor of 10 higher.

For antibiotic materials that are either inherently volatile enough for GC or derivatizable to enhance their volatility, GC–MS can provide all of the qualitative advantages of LC–MS, with much better LODs. For example, GC–MS analysis of egg samples for chloramphenicol residues [36] using negative-ion chemical ionization provided an LOD which was as good as that reported for the electron-capture detector [37] in a similar matrix.

3.5. Miscellaneous detection techniques

Several new detection modes have been reported for specific antibiotic materials. These include electrocatalytic oxidation [38], tris-2,2′-bipyridyl ruthenium(III)-based chemiluminescence [39] and chem-

ical degradation to produce an absorbing product [9]. These systems are narrow in their application and do not provide the unique information characteristic of the polarimetric or MS techniques. In addition, these methods have not demonstrated detection limits which are significantly better than the other approaches described and they therefore have not been widely applied to the analysis of food materials.

4. CHROMATOGRAPHIC ANALYSIS OF ANTIBIOTICS IN SPECIFIC FOOD MATRICES

4.1. Fish and meat

For routine screening of antibiotic residues in animal tissues, the analytical procedure utilized should provide a rapid response, it should be relatively immune from interference, and for low level monitoring it should provide low detection limits and be able to discriminate among several close structural analogues within an antibiotic class. Thus the general strategy is to extract the antibiotic from the tissue sample, isolate the antibiotic from other, co-extracted materials (proteins, fats, etc.) by either additional solvent extractions or by the use of a solid-phase extraction technique, and then use HPLC to separate and detect the various analogues within a given antibiotic system.

Interferences arise primarily from the characteristics of the food matrix. For example, animal tissues contain large amounts of protein which usually requires considerable clean-up prior to chromatographic analysis. Since many of the antibiotic materials used in veterinary applications are hydrophobic, most methods developed for use with animal tissues utilize reversed-phase cartridges for sample clean-up. However, elution of the antibiotic materials from the cartridge requires organic solvents which must be eliminated prior to chromatographic analysis. Further, reversed-phase cartridges will extract other lipophyllic materials in addition to the antibiotic compound and these must be removed in a separate procedure prior to analysis. Ikai *et al.* [40] used an amino-type prepacked cartridge for the clean-up of sulfonamide antibiotics present in animal tissues. The use of an amino cartridge significantly shortened the sample preparation time. Tissue samples were extracted with ethyl acetate and the extractant then passed through the amino cartridge. Lipophillic materials were removed from the cartridge with several hexane washes, and the sulfonamides were eluted with a 24:76 acetonitrile–0.2 *M* phosphoric acid solution. The antibiotic materials were then separated and detected using a reversed-phase column with UV detection at 272 nm.

Fig. 7 shows the reversed-phase separation of a series of ten sulfonamides separated from commercially available meats, eel and egg. The amino cartridge procedure provides effective isolation of the sulfonamide compounds resulting in a wide elution window. The complete procedure required approximately 45 min per sample. Recoveries for these antibiotic materials were in the range 74–99% with a demonstrated detection limit at the 50-ng/g level. The advantages of this procedure in terms of minimal sample preparation are not limited solely to sulfonamides but should be applicable to other antibiotic systems of similar hydrophobicity.

Wulders and Van de Lagemaat [41] have developed a Sep-Pak cartridge-based method for use in isolating tetracycline antibiotics from animal tissues. The reversed-phase nature of the Sep-Pak requires additional sample preparation and manipulation as cited earlier. Specifically, prior to chromatographic analysis, tetracyclines were eluted from the Sep-Pak cartridge with methanol and the solvent was then evaporated. Chromatographic analysis then proceeded by dissolving the residue from the solvent evaporation step in the chromatographic mobile phase. A Nova-Pak Ph stationary phase was used as the separation medium and detection accomplished by UV absorption. The need to evaporate the Sep-Pak elution solvent probably degrades sample recoveries and these authors suggested that the various tetracyclines could be recovered with 68–90% yields. The strong chromophore of the tetracyclines provided low detection limits, however, coefficients of variation for between-run analyses were high with values in the range 2–8%.

Similar results were reported by Horie *et al.* [42] in developing a method for the analysis of nalidizic acid, oxolinic acid and piromidic acid residues in fish. These synthetic antibiotic materials are used frequently in aquaculture, and residues of these materials have been reported in commercial fish tissues. Tissue samples were extracted/deproteinized under centrifugation with a methanol–phosphate

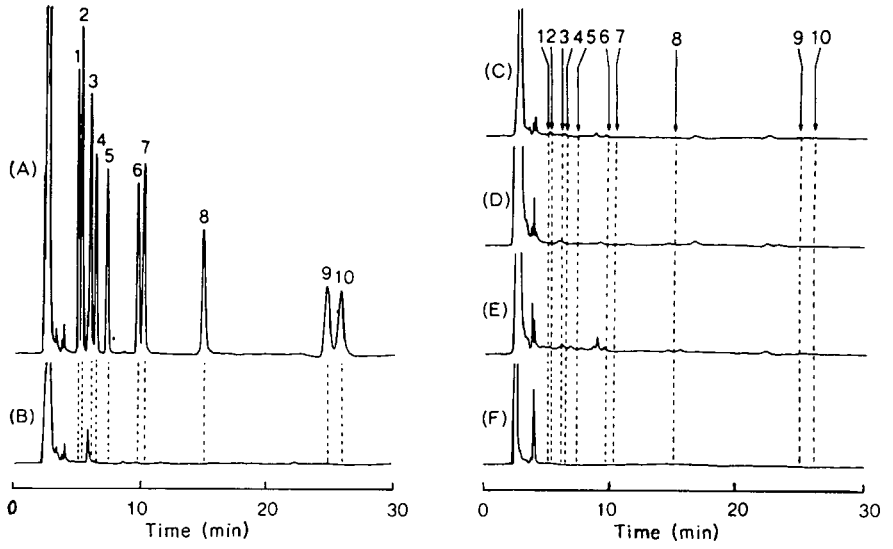

Fig. 7. HPLC separation of sulfonamide antibiotics obtained from commercially available meats, eel and egg. (A) Fortified chicken (0.5 μg/g); (B) chicken; (C) pork; (D) beef; (E) eel; (F) egg. Peaks: 1 = STZ (sulphathiazole); 2 = SDZ (sulphadizaine); 3 = SMR (sulphamerazine); 4 = SDD (sulphadimidine); 5 = SMPD (sulphamethoxypyridazine); 6 = SMMX (sulphamonomethoxine); 7 = SIZ (sulphisozole); 8 = SMX (sulphamethoxazole); 9 = SDMX (sulphadimethoxine); 10 = SQ (sulphaquinoxaline) (50 ng each). Column, Wakosil 5 C_{18} (5 μm) (250 × 4.6 mm I.D.); mobile phase, acetonitrile–0.02 *M* aqueous phosphoric acid solution (24:76, v/v); flow-rate, 1.0 ml/min; detection, 272 nm. From ref. 40.

buffer solvent. The extracted antibiotics were further isolated by Sep-Pak clean-up, eluted with methanol, the solvent was evaporated and the residues were then dissolved in the chromatographic mobile phase. A reversed-phase mode was used to separate the antibiotic compounds with either fluorescence or absorption detection of the separated materials. Excellent recoveries were reported for all three materials (>85%), with detection limits at the 10-ng/g level.

4.2. Milk and infant formula

Milk and milk products provide a measurement matrix with many potentially interfering substances for antibiotic analysis. Analytical methodologies usually contain clean-up procedures for both protein and lipophillic substances. In an effort to overcome these problems by the use of a high-efficiency separation system, a procedure has been developed based on capillary column GC for the analysis of β-lactam antibiotics (penicillins) [43]. Even with this high-efficiency separation system, extensive sample clean-up is required. The penicillins are rendered sufficiently volatile for GC analysis by methylation with diazomethane. Excellent LODs were demonstrated (< 1 ng/g), however, the need to derivatize the antibiotic limits the application of this technique in rapid-screening applications. The extensive clean-up prior to derivatization did not reduce the amount of sample manipulation required and therefore no reduction in sample preparation time was realized.

Most reported analytical procedures for antibiotic substances in milk or milk products utilize RP-HPLC as the separation mode. Long *et al.* [44] mixed C_{18} derivatized silica with the milk sample and then prepared a column from this matrix in the barrel of a syringe (10 ml). Lipophillic substances were removed from the column by washing with hexane, and a series of eight different sulfonamides were then eluted with methylene chloride. RP-HPLC with UV detection (270 nm) was then used to separate the different sulfonamide analogues. The authors found this procedure to be sensitive, free of interferences and it provided excellent recoveries of the eight sulfonamides studied.

Several recent reports have appeared which de-

scribe methodology for the separation and analysis of penicillin antibiotics in milk. These procedures utilize an acetonitrile extraction of the milk sample. Acetonitrile precipitates much of the milk protein and the precipitated protein is subsequently removed by centrifugation. Reversed-phase chromatography is used to separate the various penicillin derivatives. Junns *et al.* [45], used an initial enzymatic hydrolysis of the milk sample to enhance discrimination against potentially interfering materials by forming the penicilloate product from the β-lactam moiety in the corresponding penicillin. The terminal aldehyde species is subsequently formed using mercury(II) chloride and the penicilloaldehyde is then extracted into methylene chloride. RP-HPLC is used to separate the different penicillin analogues and detection is enhanced by derivatization with dansylhydrazine to render the penicilloaldehyde fluorescent. The major problem with this approach is the hazardous nature of the mercury salt and the difficulty in disposing of the waste from the procedure. Moats [46] used a similar acetonitrile precipitation/centrifugation step to remove protein, followed by a methylene chloride–hexane extraction to remove lipid species. The authors chromatographed the sample at pH 7 and isolated a narrow elution fraction which was then chromatographed at pH 1.96. At this pH, penicillin G provided a narrow peak which was free from interferences. The approach will work for other penicillin analogues, however, due to their different functionalities, optimum chromatographic conditions for each analogue will have to be individually determined.

A similar procedure has also been reported by Thomas [47] for the analysis of tetracycline antibiotics in milk. The milk sample is diluted with an EDTA–phosphate buffer and the antibiotic then isolated using centrifugation. RP-HPLC with a gradient of decreasing polarity (oxalic acid to methanol) is used for concentration and separation of the tetracyclines. The procedure is rapid and provides excellent detection limits which makes this procedure very advantageous for large-scale screening. Long *et al.* [48] use a solid-phase clean-up routine for tetracycline analysis in milk samples. The authors mixed the milk sample with C_{18} modified silica, packed the mixture into a column and then washed off lipid species with hexane. The tetracyclines were then eluted with an ethyl acetate–acetonitrile (1:3) mixture and chromatographed by RP-HPLC using PDA detection (365 nm). The success with which this procedure can isolate tetracycline species without interference compensates for the time required for column preparation. An alternative procedure has been described [49] which uses a series of liquid extractions to isolate tetracyclines. The milk sample is first acidified to pH 2.7 and extracted with acetonitrile. The supernatant liquid is then adjusted to pH 8.2 and the tetracyclines are extracted into methylene chloride as an ion pair with Bu_4NHSO_4. The ion pair is disrupted by extraction of the methylene chloride with acid, and the tetracyclines are separated by RP-HPLC. In general, this procedure did not provide any advantages over the others reported in terms of reduction in sample preparation or in improvement in analytical capability.

Long *et al.* [50] have also described a solid matrix isolation procedure for chloramphenicol in which C_{18} modified silica is used to isolate this antibiotic material in milk. Although minimum sample preparation is required, the analytical capabilities of the method, particularly in light of the low-level monitoring necessary for this antibiotic, are not as good as those reported for the GC methods described earlier [4].

Shaikh and Jackson [51] defatted milk samples prior to analysis for neomycin residues by centrifugation at 4°C. TFA is then added to the supernatant to precipitate proteins which are then removed by another centrifugation step. The neomycin isolated from this step is then determined by direct injection of the supernatant liquid into an RP-HPLC system. The separation mode uses ion-pair formation with TFA, followed by fluorimetric detection of the *o*-phthalaldehyde derivative (post-column derivatization) of neomycin. Excellent LODs and recoveries from spiked milk samples were demonstrated with this method.

4.3. Eggs

The analysis of antibiotic residues in eggs requires the same considerations in terms of sample deproteinization and defatting as milk. Therefore, the procedures described earlier for milk analysis can be used, with minor modification for analysis of these same antibiotic materials in eggs. A novel pro-

cedure involving dialysis isolation and on-column concentration has been developed by Aerts *et al.* [52] for the analysis of sulfonamides in egg samples. Egg samples are homogenized with a small amount

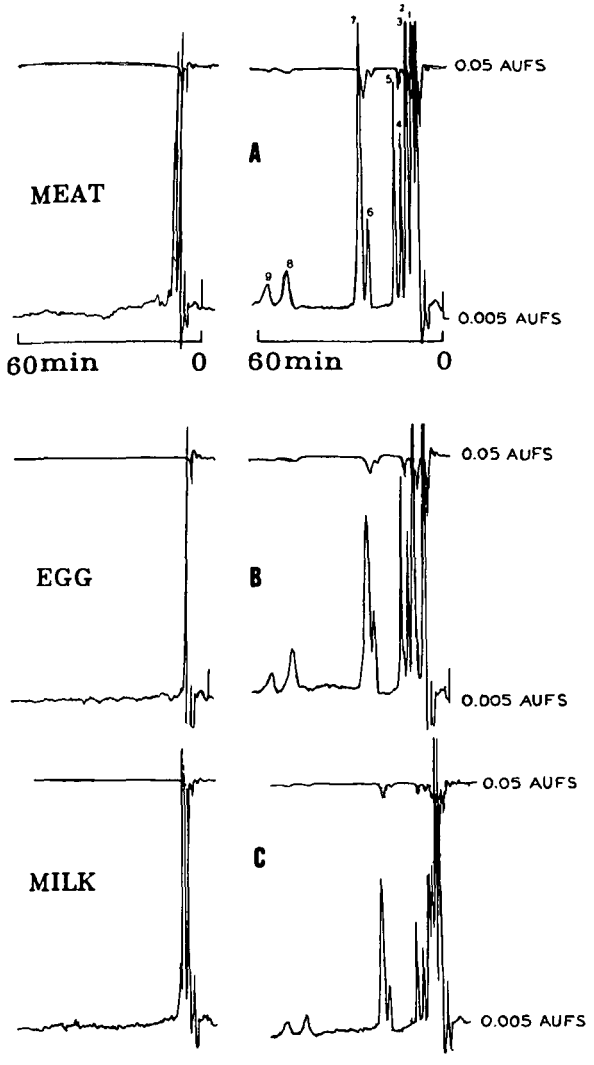

Fig. 8. Continuous-flow LC analysis of blank and spiked meat (A, 100 μg/kg), egg (B, 50 μg/kg) and milk (C, 25 μg/l) samples. Conditions: enrichment column, 60 mm × 4.6 mm I.D., XAD-4; eluent, 0.05 *M* sodium acetate (pH 4.6)–acetonitrile (82.5:17.5); analytical column, LiChrosorb RP-8; derivatization with dimethylaminobenzaldehyde (DMAB); detection at 450 nm, 0.005 a.u.f.s. Peaks: 1 = SA (sulphanilamide); 2 = STH (sulphathiazole); 3 = SD (sulphadiazine); 4 = SM (sulphamerazine); 5 = SMZ (sulphamethazine); 6 = SDX (sulphadoxine); 7 = DDS (dapsone) + STX (sulphatroxazole) + SMX (sulphamethoxazole); 8 = SDM (sulphadimethoxine); 9 = SQX (sulphaquinoxaline). From ref. 52.

of sodium azide to enhance the water solubility of the antibiotic. The aqueous sample is then placed in a continuous-flow system where it is dialysed on-line through a cellulose acetate membrane. The small antibiotic molecules pass through the membrane and are then concentrated on a short column packed with polymeric XAD-4. The concentrating column is backflushed into the analytical column where the sulfonamides are separated by RP-HPLC (C_8). Fig. 8 shows the separation of a variety of different sulfonamide analogues using this automated sample preparation, concentration and separation system. The authors found that derivatization of the antibiotic materials wth *p*-dimethylaminobenzaldehyde (post-column) enhanced their UV detection at 450 nm by a factor of approximately 2 over their inherent absorptivity at 280 nm. In Fig. 8, the level of the various sulfonamides injected was 50 ng/g and, as is evident from the data, this method provides excellent separation and detection at this level and below.

With respect specifically to egg samples, this method provides significant advantages over others in that problems with emulsification are minimal. Fig. 9 shows the separation of two different sulfonamides separated from eggs using this automated method. The excellent signal-to-noise ratio obtained for sulfaguanidine suggests that this method should be able to detect several of the sulfonamides at the low ng/g level, or below. In general, one of the main advantages of this method lies in its potential for wide applicability. The realm of sulfonamides investigated with this method spanned the range from polar to relatively non-polar, and from weakly acidic to basic. This range of chemical functionalities and characteristics also encompasses many other antibiotic materials of physiological concern and the method should therefore be extendable to the analysis of these antibiotic materials in similar matrices.

4.4. Honey

As a sample matrix, honey does not present as many challenges as, for example, milk or eggs. Diaz *et al.* [53] have developed a procedure for the analysis of tetracyclines and sulfathiazole which involves simple dissolution of the honey sample in a mixture of acetonitrile–water (10:90) prior to analysis. The

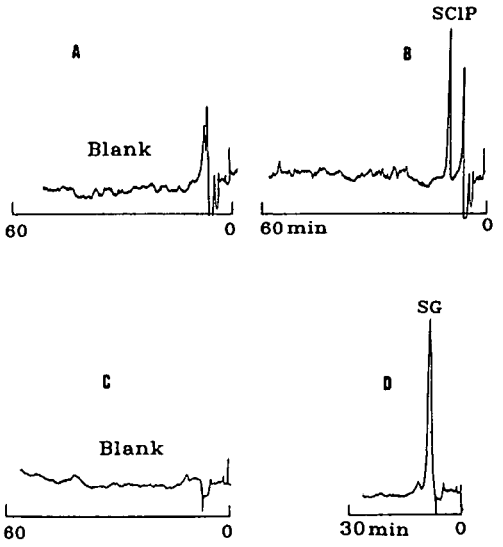

Fig. 9. Continuous-flow LC analysis of egg samples spiked with SCP (sulphachlorpyrazine) (100 μg/kg, A and B) and SG (sulphaguanidine) (50 μg/kg, C and D). Conditions for (A) and (B): enrichment column, 60 mm × 4.6 mm I.D., XAD-4; eluent, 0.05 *M* sodium acetate (pH 6.85)– acetonitrile (87.5:12.5, v/v); analytical column, C_p TM-Spher C_{18}; derivatization with DMAB; detection at 450 nm, 0.005 a.u.f.s. Conditions for (C) and (D) are the same as (A) and (B) except the eluent is water–acetonitrile–acetic acid (97:2:1, v/v/v). From ref. 52.

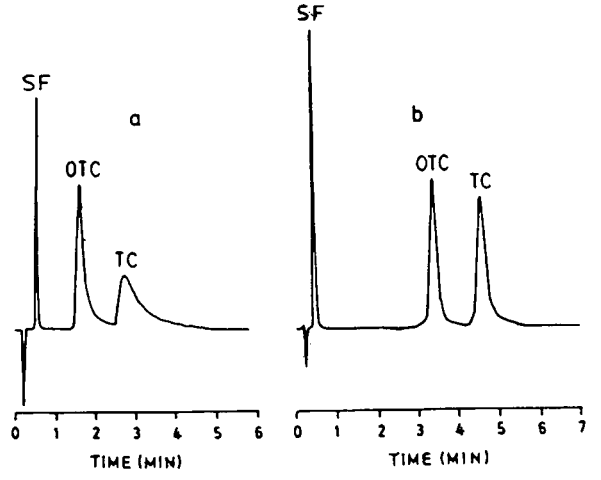

Fig. 10. (a) HPLC separation of a standard sample containing 164 ng of SF (sulfathiazole), 668 ng of OTC (oxytetracycline) and 578 ng of TC (tetracycline); mobile phase 0.01 *M* oxalic acid–acetonitrile (85:15). (b) HPLC separation of a standard sample containing 156 ng of SF, 535 ng of OTC and 622 ng of TC; mobile phase 0.01 *M* oxalic acid, 0.01 *M* SDA (sodium dodecyl hydrogensulfate)–acetonitrile (70:30). From ref. 53.

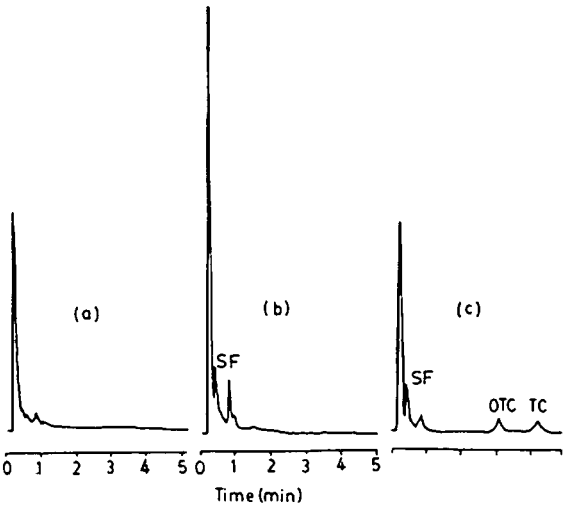

Fig. 11. HPLC separation obtained from (a) honey sample with no appreciable amount of analytes, (b) honey sample with appreciable amount of SF and (c) honey sample fortified with 10.4 μg/g SF and 23.1 μg/g OTC and TC. Chromatographic conditions are the same as in Fig. 10 (b). From ref. 53.

dissolved sample is filtered by a syringe filter and the filtrate then injected directly into the HPLC system. Micellar reversed-phase chromatography is used to separate the antibiotic species with dodecyl hydrogensulfate as the mobile phase additive. Sensitive detection was accomplished photometrically at 285 nm.

Fig. 10 clearly demonstrates the advantages of the micellar mode. In (a), severe peak tailing is evident for the two tetracycline species. In (b), an anionic micellar mobile phase was used which provided a significant reduction in peak tailing. Fig. 11 shows the application of this optimized separation system to the analysis of a honey sample spiked with these three antibiotics. The chromatographic system provides excellent separation with only minimal sample preparation. However, the detection mode utilized by these authors is not as sensitive as others reported for these antibiotics and significant improvements could be realized by application of these other modes to the analysis of tetracyclines.

5. CONCLUSIONS

As has been discussed, the monitoring of food materials for antibiotic residues is an area of in-

creasing concern and importance due to the potential impact on human health. Large-scale screening applications require methods that are rapid, accurate, provide low detection limits and are free from interference. The problem is further complicated by the wide range of chemical functionalities and modes of operation exhibited by the antibiotic materials of physiological significance in use today. As demonstrated, chromatographic methods provide many of the advantages necessary for screening applications. Judicious choice of sample preparation method, separation mode and detection strategy can provide significant immunity from problems associated with the food matrix.

Among the methods surveyed, RP-HPLC is used extensively for the analysis of many antibiotic systems as it combines relatively high separation efficiencies with low detection limits. Among the different detection modes utilized for antibiotic analysis, polarimetric detection has the potential to provide extremely selective detection of most antibiotic materials, and this selective response can minimize many of the constraints placed upon the separation system by the sample matrix. Although many of the separation modes used for antibiotic analysis are standard, separations based on capillary electrophoretic methods have much potential in the field of antibiotic analysis. Future investigations are needed to extend the generality of these techniques and expand their use into the field of food analysis.

6. ABBREVIATIONS

CE	Capillary electrophoresis
CMC	Critical micelle concentration
DMF	Dimethylformaldehyde
GC	Gas chromatography
HFBA	Hexafluorobutylacetate
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
LOD	Limit of detection
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
PDA	Photodiode array
RP-HPLC	Reversed-phase high-performance liquid chromatography
RP-TLC	Reversed-phase thin-layer chromatography
SDS	Sodium dodecyl sulfate
SFC	Supercritical fluid chromatography
TAA	Tetraalkylammonium
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TMS	Trimethylsilane
TSP-MS	Thermospray mass spectrometry
UV	Ultraviolet

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Review

Chromatographic methods of analysis for penicillins in food-animal tissues and their significance in regulatory programs for residue reduction and avoidance

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ABSTRACT

Chromatographic methods for penicillin analysis in animal tissues play a significant role in the regulation of the use of these drugs in livestock production. Regulatory agencies rely on data generated from these methods to establish withdrawal times and to determine whether presumptive positive tissue samples from slaughtered animals intended for human consumption contain violative levels of penicillins to necessitate regulatory action. The need to develop sensitive, accurate, and reliable methods to support regulatory programs is examined together with emerging techniques that could be taken advantage of to improve the sensitivity and usefulness of current chromatographic methods for tomorrow's regulatory agency.

CONTENTS

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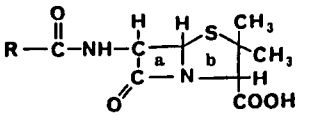
1. INTRODUCTION

Penicillins and cephalosporins belong to the group of antimicrobial drugs commonly referred to as β-lactam antibiotics. In this review, only penicillins will be discussed. Penicillins contain the basic β-lactam ring (a) coupled to a thiazolidine ring (b) (see Fig. 1) to form 6-aminopenicillanic acid, generally referred to as the "penicillin nucleus". To the 6-aminopenicillanic acid moiety are attached side-chains (R), which determine the stability and the spectrum of antimicrobial and pharmacological activities of the different β-lactams. Penicillins are classified as either natural or synthetic; they may also be classified on the basis of their resistance to gastric acidity and penicillinases or as broad spectrum. Penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin), produced metabolically by molds of *Penicillium notatum* and *Penicillium chrysogenum*, are the natural penicillins. The amino (ampicillin, amoxicillin) and isoxazolyl (oxacillin, cloxacillin, dicloxacillin and methicillin) derivatives of penicillin are semi-synthetic. Phenethicillin and penicillin V are acid-resistant, the isoxazolyl penicillins are penicillinase-resistant while ampicillin, amoxicillin, hetacillin, carbenicillin and ticarcillin belong to the broad spectrum group of penicillins. Fig. 1 shows the basic penicillin structure and the chemical structures of the other penicillins commonly used in veterinary medicine for food-producing animals.

Penicillins are administered by various routes for the control of mastitis in dairy cows and for treating infections of several other tissues in cattle, such as the urinary, gastrointestinal and respiratory tracts. They may also be added to swine feed and to the drinking water of poultry. Generally, penicillin drug formulations are designed to be used according to the approved label dose specifications, which include the dosing rate and frequency (pharmacokinetic values) as well as the route of administration. The veterinary medical profession attends to many species of animals, with highly significant variations in physiology and pathology. Even within the same species, considerable variation might occur in pharmacokinetic values when sick animals are treated [1]. As a result, it is virtually impossible to have an approved drug labelled for every disease condition in animals, and extra-label drug use (*i.e.*, use deviating from the drug label's directions on route of administration, dosage, duration of treatment, species, or indicated disease condition) is therefore a reality of veterinary practice. Penicillins are also administered in lower dosages (prophylactically) to prevent diseases in exposed animals and at subtherapeutic levels in animal feeds to improve feed efficiency and growth of livestock.

The legitimacy of drug use, especially at extra-label dosage rates in food-producing animals, has been questioned by several groups, including those interested in animal welfare and animal rights, those concerned about the safety of the individuals administering the drugs, and those concerned about the perceived potential for human health risks from drug residues in tissues. Health risks usually cited include the development of resistant bacteria by gene transfer to the progeny via the chromosome and gene transfer on R-plasmids in animals treated with penicillin [2], the development of penicillin-resistant pathogenic bacteria in the consumer as a result of increased intake of penicillin residues in animal tissues, and the development of hypersensitivity in some individuals to this drug. Most of the documented cases of allergic responses to the ingestion of penicillin-contaminated food have been linked to milk and milk products [3], but a few cases of penicillin-implicated allergic reactions from meat [3–6] are known (Table 1). In addition, survey



BASIC PENICILLIN NUCLEUS

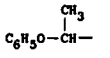
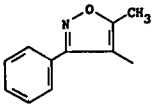
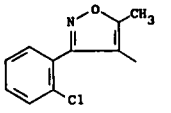
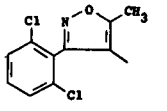
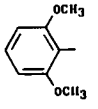
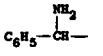
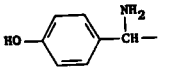
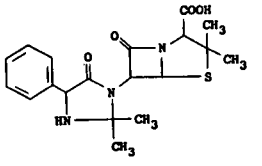
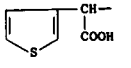
CLASSIFICATION	R	GENERIC NAME
NATURAL	$C_6H_5OCH_2-$	PENICILLIN G
NATURAL, ACID RESISTANT	$C_6H_5CH_2-$	PENICILLIN V
SYNTHETIC, ACID RESISTANT		PHENETHICILLIN
SYNTHETIC, ISOXAZOLYL		OXACILLIN
		CLOXACILLIN
		DICLOXACILLIN
		METHICILLIN
SYNTHETIC, BROAD SPECTRUM		AMPICILLIN
		AMOXICILLIN
		HETACILLIN
		CARBENICILLIN
		TICARCILLIN

Fig. 1. Chemical structures of penicillins commonly used in veterinary medicine for food-producing animals.

TABLE 1

DOCUMENTED CASES OF PENICILLIN-INDUCED ALLERGIC REACTIONS FROM MEAT

Year	Comments	Symptoms
1972	Male victim had previously been treated with penicillin. A pig was slaughtered 3 days after penicillin injection. Victim (a butcher) consumed one bite of meat from the pig and suffered the reaction [3]	Pruritus on face and fingers, generalized rash
1972	A penicillin-allergic patient suffered an acute allergic reaction in West Germany after ingesting freshly processed pork. The meat, which was found to contain 0.3–0.45 U/g penicillin residues, had been eaten from a pig that had been treated with therapeutic levels of penicillin 3 days before slaughter [4]	Angioedema and pruritus
1980	A 45-year-old American female, with prior history of penicillin allergy, suffered an anaphylactic reaction following the ingestion of a frozen meat dinner. Beef was found to contain residues of penicillin [5]	Generalized pruritus, difficulty swallowing, speaking and throat tightness
1981	Two females, aged 37 and 42 years, who had prior history of penicillin allergies, got sick after eating raw pork from a pig that was experimentally injected with 3 million U of penicillin for 3 days and slaughtered. The meat was fed to a group of nine volunteers which included the two female victims [3]	Pruritus on face
1989	A 35-year-old female from Calgary suffered allergic reaction after eating about 4 ounces of roast beef purchased from a local grocery store. The roast was found to contain penicillin G and sulpha residues. Producer did not follow prescribed withdrawal times [6]	Patient broke out in hives and became very sick

studies conducted by regulatory agencies continue to detect penicillin residues in animal tissues at slaughter [7–10]. Why are penicillin residues being detected in tissues of food-producing animals?

Several papers dealing with the reasons for the incidence of antimicrobial residues in meat have been published [3,11,12], including the recent report by Van Dresser and Wilcke [8] on the state of residues in food animals in the USA. Observations made over the years at the Health of Animals Laboratory from the analysis of presumptive positive tissue samples [7] and field operation investigations in Canada report similar findings. According to the US report, penicillin, streptomycin, neomycin, oxytetracycline and sulphamethazine are the most frequently detected residues in animal tissues in the USA. Residues found were predominantly in cows, veal calves and market hogs (barrows and gilts). The reason most frequently cited by field investigators for drug residues was failure to observe withholding time for the drug; in over 80% of the cases for which responsibility was determined, the producer was considered the responsible party.

Pharmacokinetic data developed by manufacturing companies for drug approval are generated for drugs use at the label dose only. For food-producing animals, it is required that, prior to slaughter, an animal be withdrawn from treatment for a recommended period. There are no defined withdrawal periods, however, when penicillin formulations are used in an extra-label manner. It is believed that the unavailability of recommended withholding periods for penicillin use at extra-label doses has largely contributed to the presence of residues in food-producing animals.

These observations and the fact that the livestock industry is a multi-billion dollar industry with substantial global trade ramifications have prompted regulatory agencies in countries throughout the world to take measures not only to safeguard the consumer against any health risks from contaminated meat, but also to ensure that trade restrictions which affect the survival of the industry do not occur.

It is in support of this regulatory process that the analysis of penicillin G residues in animal tissues becomes important. This paper reviews the chromatographic methods of chemical analysis that have so

far been developed for the determination of penicillin residues in animal tissues and examines the role they play in regulatory programs in helping to allay the fears and concerns of the public. Also, anticipated trends in improvements to currently available methods developed for the analysis of penicillin residues in animal tissues are examined.

1.1. Development of methods for the assay of penicillins

Methods are developed for the analysis of penicillins in blood [13–20], urine [15,18,21] and other body fluids [22–25] and tissues primarily to obtain pharmacological and pharmacokinetic data. With biological fluids [26–40] in particular, this information is required in order to establish the adequacy of therapy, establish whether a sick animal fed a β-lactam is, in fact, absorbing the drug from the digestive tract, establish therapeutic levels of drugs in various body compartments, etc. Therefore, the development of analytical methods for the assay of penicillins in biological fluids is very useful to the clinician. For the consumer of food-producing animals, however, it is important that veterinary drug residues are not allowed to occur in the animal tissue intended for consumption. Methods developed for the analysis of penicillin in animal tissues provide the necessary data base required for defining withdrawal times (tissue depletion), and for determining whether presumptive positive tissue samples contain sufficient levels of a defined veterinary drug to necessitate regulatory action. The development of methods for the assay of penicillins in animal tissues is therefore of primary significance to agencies responsible for the regulation of veterinary drugs in food animals.

1.2. Classification of methods for the analysis of penicillins in animal tissues

Analytical methods for the detection of penicillin residues in animal tissues may be classified into two broad groups: (a) those based on immunological and microbiological techniques which respond to groups of penicillins but do not discriminate among them (screening methods), and (b) those which are compound-specific, involving chemical or physical separation of the specific drug from other components for the detection, identification and discrimination among different penicillins (chromatographic methods).

2. SCREENING METHODS FOR PENICILLIN RESIDUES IN ANIMAL TISSUES

Screening methods for the detection of penicillin residues in animal tissues are usually very sensitive to penicillin G, with detection sensitivities in the μg/kg concentration range, and they provide fairly reliable test results. The procedures for conducting the screening tests are relatively simple and inexpensive, and require very simple equipment. However, because they lack compound specificity and are, at best, semi-quantitative, they have traditionally been used as the method of choice when only a qualitative decision is needed as to whether the tissue sample is contaminated with a microbial inhibitor or not. These screening methods include the classical microbial inhibition tests, such as the Swab test on premises (STOP) [41], the calf antibiotic and sulpha test [42], and the live animal Swab test [43], the inhibition tests which use colorimetric reactions, such as the Brilliant Black reduction test [44] and the Charm farm test [45], and the immunological assays, such as the Charm test II receptor assay [46] and enzyme-linked immunosorbent assays [47].

Until 1977, when the first application of chromatographic analysis for penicillin residues in tissues was published [48], all research that had hitherto been done on penicillin residues in animal tissues had used a screening test. Even today, these methods are still being used. In fact, more than 80% of what we know today about penicillin residues in animal tissues is as a result of the applications of these screening tests to animal studies [49–69]. In 1981, Johnston *et al.* [41] published a screening method, the STOP test, for the detection of antibiotic residues in meat and poultry tissues with a detection sensitivity of 0.0125 μg/g for penicillin G. This test has been used in most abattoirs in Canada and the USA for the past decade. The STOP test requires overnight incubation, so test results are not ready until the following day. With increased consumer demand for more testing and industry demands for quicker turn-around times for sample analysis, it has become prudent to find alternative or more suitable tests to the STOP test for high-volume

TABLE 2

THIN-LAYER CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES

ACN = Acetonitrile; TZC = 2,3,5-triphenyltetrazolium chloride.

Penicillin formulation and animal experiment conducted; tissue matrix analyzed	Extracting/deproteinizing solvent; assay medium; developing solvent	Detection method and sensitivity of method	Authors and ref.
Procaine penicillin G, sodium penicillin G, spiked into cooked meats, freeze-dried chicken and hamburger; metabolites formed after cooking above spiked tissues were methylated and analyzed	Methanol; silica gel; ethyl acetate–acetic acid–water (8:1:1)	Autoradiochromatography with liquid scintillation counting	DePaolis *et al.* [48]
Ampicillin and penicillin G in imported, casualty and post-mortem animals in the UK; pigs, sheep, beef, veal, goat, kidneys, livers and muscles	ACN–water (9:1); silica gel; toluene–ethyl acetate–diethyl ether–acetic acid (80:10:10:0.75)	Bioautography using *Bacillus cereus* and TZC. Sensitivity for penicillin G (0.01 I.U.)	Smither [65]
Procaine penicillin G (300 000 I.U.) injected intramuscularly to Holstein calves and Yorkshire pigs; ampicillin (200 mg) also injected to similar animals	Methanol; silica gel plates	Bioautography using *Micrococcus luteus* and *Bacillus subtilis*. Developing reagent was TZC	Yoshimura *et al.* [75]
Penicillin B, penicillin V standards spiked into kidney, liver and muscle tissue for method development; diagnostic samples	Methanol; silica gel and cellulose plates; ACN–chloroform–1-propanol–impregnating liquid (16:20:27:16)	Bioautography using *Bacillus subtilis*. Sensitivity to penicillin G (0.08 I.U./g)	Neidert *et al.* [76]
Penicillin G in spiked pork and beef tissue; diagnostic samples: avian, equine, bovine, porcine livers, kidneys and muscles	Methanol; silica gel; chloroform–methanol–acetone–glycerine (49:30:20:1)	Bioautography using *Bacillus subtilis*. Sensitivity to penicillin G (0.03 μg/g)	Salisbury *et al.* [7]

TABLE 3

GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUE

Tissue matrix analyzed; method of analysis, compounds analyzed; analytical sample size	Deproteinizing solvent; extraction method	Detection method and sensitivity	Authors and ref.
Spiked bovine muscle, kidney, liver and adipose tissue; temperature-programmed injector vaporization, DB-1 fused-silica capillary column; penicillins G and V, methicillin, oxacillin, cloxacillin, dicloxacillin, nafcillin, 25 g homogenized tissue	Acetonitrile (pH 4.0–4.4); extract partitioned into organic aqueous solvents including dichloromethane, water, light petroleum–phosphate. Residue cleaned up on SAX cartridge, dichloromethane–aqueous extract, methylation, diol cartridge clean-up	GC with thermionic (nitrogen–phosphorus) detector, detection limits < 2 ng/g for all β-lactams	Meetschen and Petz [80]

sample screening, especially when other more sensitive screening tests capable of providing test results in a few hours are available today.

3. CHROMATOGRAPHIC METHODS FOR PENICILLIN RESIDUES IN ANIMAL TISSUES

These methods of analysis include thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and electrophoresis. Tables 2–5 catalogue the various chromatographic methods that have so far been developed for the analysis of penicillin residues in food-animal tissues.

3.1. Criteria for chromatographic methods of analysis in regulatory programs

For a chromatographic method developed for the analysis of penicillin in animal tissue to be useful in the regulatory program, it should meet reasonable standards of precision, accuracy, specificity, and, above all, it must be practical to use. Since it is probably intended to be used for the analysis of a large number of diagnostic samples with expected rapid turn-around times, the method should be repeatable and reproducible, and it should not take too long before test results are obtained. Typically, an analytical method for the determination of a veterinary drug is considered to be accurate if the means of the observed recoveries from fortified samples are 80–110% of the added marker residue at levels $\geqslant 0.1$ μg/g or tolerance levels, if the latter are defined. Below 0.1 μg/g or tolerance, mean observed recoveries from fortified samples should be between 60 and 100%. The precision of the method should be such that the intra-laboratory relative standard deviation (R.S.D.) (repeatability) of the assay does not exceed 10% at the tolerance level defined for the residue of interest [70]. It should have a sensitivity well below the tolerance level defined for the veterinary drug of interest and should be capable of confirming the presence of the drug at the violative level. In addition, residues from tissues of food-producing animals that have been experimentally administered with the drug of interest (to produce physiologically incurred or dosed tissue samples) have to be determined to ensure that the initial extraction procedures incorporated into the method, which are usually developed with spiked samples, are adequate to recover residues from true samples.

Determinative chromatographic procedures such as TLC and HPLC, which use UV–VIS, fluorescence, and radioimmunoassay detection, and GC, which relies solely on the use of retention data for identification, are not deemed to be specific enough to support regulatory action. In contrast, chromatographic methods that rely for detection and identification on total molecular configuration, such as infrared spectroscopy (IR) or mass spectrometry (MS), are considered to be specific enough to support regulatory action. The various chromatographic methods, listed in Tables 2–5 and developed for the analysis of penicillin residues in animal tissues, will therefore be reviewed with respect to how useful they are in providing an analytical support service for regulatory programs.

3.2. Isolation techniques for the chromatographic analysis of penicillins in tissues

When procaine penicillin G is administered to a food-producing animal, it is usually absorbed very rapidly. Its distribution is essentially limited to the extracellular fluid space. Protein binding, which is primarily to albumin, is estimated to be 60% for penicillin G, 20% for ampicillin and amoxicillin, and in excess of 90% for the isoxazolyl penicillins. Even at these extensive protein binding rates, significant concentrations of penicillins can still be found in liver, kidney, intestine, bile, lymph and semen [71]. Penicillin is eliminated very rapidly from the body, mainly by the kidney. For example, after injection of an aqueous solution of penicillin G, 60–90% may be recovered within 1 h in the urine. The absorption and elimination characteristics of penicillins in food animals indicate that penicillins would be in a non-covalently bound or unbound state and probably at very low concentration (ng/g) levels in the tissues intended to be analyzed.

The animal tissue samples from which the penicillins are to be determined are typically complex mixtures, composed of a wide variety of carbohydrates, proteins, lipids, etc., as well as penicillins (bound or unbound) if the animal has been so treated. Besides the deleterious effects these materials may have on the chromatographic components,

TABLE 4

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUES

ACN = Acetonitrile; RP = reversed-phase; K = kidney; L = liver; M = muscle.

Tissue matrix analyzed; method of analysis; compounds analyzed; analytical sample size	Deproteinizing solvent; extraction method	Detection method and sensitivity	Authors and ref.
Cooked meats and freeze-dried chicken and hamburger; HPLC of methylated penicillins and metabolites; procaine penicillin G, sodium penicillin G, degradation products (metabolites) formed after cooking meat	Methanol	UV detection at 254 nm, MS, NMR	DePaolis *et al.* [48]
Spiked and incurred muscle, kidney and liver tissues from beef and pork; isocratic C_{18} RP chromatography; penicillin G and cloxacillin; 25 g homogenized tissue	ACN; extract was treated with dichloromethane, light petroleum + ACN, phosphate buffer, light petroleum, saturated ammonium phosphate and ACN	UV detection at 220 nm; 50 ng/g penicillin G, 20 ng/g cloxacillin	Moats *et al.* [83]
Spiked muscle, kidney and liver; on-line pre-concentration and manual column switching onto C_{18} RP column under isocratic conditions; 10 g homogenized tissues	Sulphuric acid, sodium tungstate; extract was cleaned up on basic alumina then Sep-Pak C_{18}	UV detection at 220 nm; 50 ng/g	Terada *et al.* [78]
Spiked fish muscle tissue; isocratic C_{18} RP chromatography; ampicillin; 10 g minced tissue	Methanol; extract was treated with propanol, followed by Florisil cartridge clean-up	UV detection at 222 nm; 30 ng/g	Nagata *et al.* [74]
Bovine tissue; initial RP chromatography, fraction collection and rechromatography of fractions by thermospray LC–MS; ampicillin, cloxacillin, penicillin G; 2 g tissues	Sonicate in ACN; methanol, water, ultrafiltration (10 000 rel. mol. mass filter)	Thermospray LC–MS	Tyczkowska *et al.* [77]
Spiked and incurred porcine and bovine (M, L, K) tissues; isocratic C_{18} RP chromatography. Penicillin G with penicillin V as internal standard; 5 g homogenized tissue	Sulphuric acid, tungstate; water extract cleaned up on C_{18} Bond Elut cartridge, eluate derivatized	UV detection at 325 nm; 5 ng/g penicillin G	Boison *et al.* [72]
Spiked and incurred bovine (M, L, K) tissues; C_{18} RP chromatography; penicillin G with penicillin V as internal standard; 5 g homogenized tissues	Sulphuric acid, tungstate; water extract cleaned up on C_{18} Bond Elut cartridge eluate analyzed directly	Thermospray LC–MS; 5 ng/g penicillin G	Boison *et al.* [79]
Cloxacillin standards in typical extraction solvents: methanol, ACN, water, ethanol, 2-propanol and mixtures of these (no tissue matrix involved)		Thermospray LC–MS	Tyczkowska *et al.* [81]
Farmed salmon, penicillin G		Thermospray, ionspray, particle beam, moving belt LC–MS	Pleasance *et al.* [118]

TABLE 5

ELECTROPHORETIC METHODS FOR THE ANALYSIS OF PENICILLIN RESIDUES IN ANIMAL TISSUES

AMP = Ampicillin; AMOX = amoxicillin; ACN = acetonitrile; CLOX = cloxacillin; DCLOX = dicloxacillin; METH = methicillin; PHEN = phenethicillin; K = kidney; L = liver; M = muscle.

β-Lactams analyzed and experimental method; analytical sample size	Extraction method	Detection method and sensitivity	Authors and ref.
AMP, AMOX, penicillin G, CLOX, METH, PHEN, penicillin V spiked into muscle, liver and kidney tissues, potential of 20 V/cm applied to electrodes, examined migration rates in agar and agarose at pH 6 and 8; 5 g tissue samples	Add ACN–deionized water (9:1), centrifuge at 38 000 *g* for 10 min at 4°C, evaporate to dryness. Dissolve in ACN–ethanol–water (0.5 ml)	Bioautography with *Bacillus cereus* and *M. luteus*. AMP (0.002), AMOX (0.002), penicillin G (0.002–0.005), CLOX (0.1–0.2), METH (0.005–0.2), PHEN (0.002–0.01), penicillin V (0.005–0.01)	Smither and Vaughan [84]
AMP, penicillin G in imported, casualty, post-mortem animals in the UK (M, L, K) from goats, sheep, veal, beef and pigs, potential gradient of 20 V/cm applied to electrodes	Add ACN–water to freeze-dried chicken (L) and pig (K) as in ref. 84	Bioautography using *Bacillus cereus* and *Micrococcus luteus*	Smither [65]
UK-produced meat and imported meat; same as reference above	Same procedure above	Bioautography using *Bacillus cereus* and *M. luteus*	Smither *et al.* [85]

such as the injectors, pumps, detectors, column packing material, etc., their presence will frequently interfere with the separation of the analyte of interest. Consequently, once a representative sample size has been defined and the analytical sample to be used for the analysis has been obtained, chromatographic methods of analysis for penicillins in animal tissues require that the penicillins be freed first of all from protein binding, followed by some form of sample preparation and/or purification procedures to isolate the drug of interest prior to chromatographic analysis and detection. These procedures have included solvent extraction/partitioning of the penicillins between aqueous and organic solvents, or the adsorption of penicillins onto suitable adsorbents (C_8, C_{18}, ion-exchange) and eluting them with suitable solvents.

3.3. Sampling animal tissues for penicillin residue analysis

Penicillins are usually distributed fairly homogeneously in non-injection site muscle, liver, kidney, interstitial, and biological fluids. This is, however, not the case with the distribution in injection site muscle mass, where there is a tendency to find localized deposits of penicillin. There is, therefore, a need to define a minimum sample size that must be taken from a bulk tissue in a food-producing animal to provide a representative sample of the bulk sample. When the chromatographic method for the determination of penicillin residues in animal tissues was recently developed [72], the authors had established that, for a detection limit of 5 ng of penicillin G per gram of tissue (*i.e.*, 0.626 ng of penicillin injected onto the column), the minimum tissue mass (analytical sample) fortified with penicillin that had to be extracted was 5 g. With this analytical sample size defined, an experiment was conducted to define the minimum sample size of organ or non-injection site muscle that had to be taken from tissues of experimental steers injected with procaine penicillin G to provide homogeneous representative samples using the criteria that repeatable analytical data must have R.S.D.s of 15% or less. It was found that the R.S.D.s obtained from the analysis of eight individual 5-g samples subsampled from homogenized 40-g incurred muscle, kidney, or liver taken

from various portions of the bulk tissue were $<15\%$. Application of the *t*-test to the calculated means of the eight replicate results from each 40-g lot showed no statistically significant difference ($P = 0.05$) from the means of multiple analysis from other 40-g lots. Similar observations were made when sample sizes of 60–100 g were homogenized and eight 5-g samples were subsampled and analyzed by HPLC. However, when sample sizes $\leqslant 20$ g were sampled from the bulk material and subsampled for analysis, means from quadruplicate analysis were found to be statistically different from lot to lot and R.S.D.s ranging from 20 to 60% were obtained. In the research laboratory at the Health of Animals Laboratory, therefore, sample sizes of approximately 40 g from the bulk material are routinely used for homogenization, before subsampling 5-g amounts for chromatographic analysis for penicillin G. For injection site analysis, it was found that tissue mass in excess of 400 g must be processed prior to subsampling for analysis in order to obtain homogeneously representative samples [73]. In addition, if samples are stored prior to analysis, they have to be sampled into 40-g lots and stored unblended. These stored samples should be blended only on the day of the analysis (to prevent accelerated degradation of penicillins) after they have been allowed to thaw at room temperature.

3.4. Protein precipitation

Protein precipitation to free non-covalently bound penicillins and remove macromolecules from the sample to be chromatographed has been achieved either by direct treatment with methanol [7,48, 74–77], acetonitrile [55,65,77], sodium tungstate and sulphuric acid [72,78,79], acidified acetonitrile (pH 4.0–4.4) [80] or by ultrafiltration [77]. With solvents, deproteinization is usually done after homogenizing the tissue to provide an increased surface area for contact with the deproteinizing reagent to facilitate the process and to provide a favorable extraction medium for the analyte. If the stability of the penicillins in the solvents that have been used for the initial extraction and/or deproteinization step is considered, it becomes apparent that methanol, which is known to degrade penicillins most extensively [81,82] and most rapidly [40] and would be the least appropriate solvent to be used for this purpose, has been used extensively [7,48,74–77]. Ultrafiltration involves the use of molecular mass cut-off filters to remove proteinaceous material. The only drawback with this technique is that not all low-molecular-mass proteinaceous material is removed and other interfering materials may pass through the filter as well. In addition, penicillins may be lost if there is any significant protein binding. The organic extract obtained at this point may either be used directly for chromatographic analysis or subjected to further purification.

3.5. Solvent extraction of penicillin residues from animal tissues

An alternative approach for isolating penicillins from tissues combines deproteinization with a solvent extraction step using either water or an organic solvent under acidic conditions. At the Health of Animals Laboratory, sodium tungstate, sulphuric acid and water are used to deproteinize and extract penicillins from tissues [72]. This technique has been found to work extremely well for isolating penicillins from eggs, plasma, tissue juices, milk, urine, yogourt and cottage cheese. This technique provides a more selective extraction of the sample matrix and offers more scope for the separation of the penicillins from endogenous material, particularly for the polar and acid-labile penicillins, such as ticarcillin and penicillin G. With this approach, proteins in the sample will usually be denatured and left at the liquid–liquid interface. With water as the extracting solvent [72,78], only the water-soluble polar components are partitioned from the tissue matrix into the aqueous phase. In contrast, when an organic solvent which may have a wider distribution of partition constants for tissue components than water is used for extraction [74,80], more endogenous components (both polar and non-polar), in addition to the drug of interest, are extracted. In such cases, further clean-up is usually warranted. The commonest approach to further clean-up of these organic extracts is to back-extract the penicillins into buffered solutions, as was done by Moats [83].

3.6. Further sample clean-up: adsorption and elution techniques

Most tissue extracts treated according to the

methods detailed in sections 3.2.–3.5. would still contain too much, and probably too many, of the other co-extractants (endogenous and exogenous material). The presence of co-extractants in the final extract usually has deleterious effects on the chromatographic components. Co-extractants increase chemical background noise in the detector and make it impossible to determine trace level concentrations of the veterinary drug. They will usually also interfere chromatographically with the drug of interest. Further purification of the penicillins from co-extractives must therefore be conducted.

As a prelude to trace level chromatographic analysis, further purification of the tissue extract, coupled with some form of concentration of the analyte, will have to be conducted at this point. A review of the literature shows that, for the penicillins, this has been achieved by solid-phase extraction and ion-exchange techniques.

3.6.1. Solid-phase extraction[a]

Meetschen and Petz [80] used diol cartridges to further clean-up extracts obtained after solvent extraction and ion-exchange techniques. Terada *et al.* [78] used Sep-Pak C_{18} cartridges after the initial extract had been passed through an alumina cartridge by gravity. Boison *et al.* [72] used Bond Elut C_{18} cartridges instead of the Sep-Pak cartridges used by Terada *et al.* [78]. This is because it was discovered during the method development by the former authors that the regular Sep-Pak C_{18} cartridges had $<14\%$ carbon loading whereas the Bond Elut cartridges had a carbon loading of 18%. It was also found that a minimum carbon loading of 17% in the packing bed was required in order to obtain $>70\%$ recovery of penicillin from the reversed-phase solid-phase extraction column. It is therefore important to consider this critical control point in any method development which includes a solid-phase extraction cartridge clean-up step, if penicillin losses are to be kept to a minimum. Table 6 compares the UV detector responses on tissue extracts for penicillins G and V obtained according to the method of Boison *et al.* [72] from muscle tissues fortified with 200 and 300 ng/g penicillins G and V, respectively. Recoveries from cartridges with a carbon loading of $\geqslant 17\%$ were in the order Sep-Pak plus > Bond Elut > SPE* for both penicillin G and V. Recovery from SPE* and the regular Sep-Pak C_{18} cartridges ($\leqslant 14\%$ carbon loading) were significantly lower than for the cartridges with $\geqslant 17\%$ carbon loading. Also, Terada *et al.* [78] used a pre-column to concentrate the dilute penicillin extract from the Sep-Pak cartridge. The concentrated extract was then loaded onto an analytical LC column using a manual switching device, and fed into a UV detector. Unless this column switching stage is automated, this technique would be heavily analyst-dependent and be unattractive for use in a regulatory laboratory.

[a] Any reference to a commercial product in this review does not imply an endorsement or rejection of the product.

4. CHROMATOGRAPHIC ANALYSIS OF PENICILLINS FROM ANIMAL TISSUE

Tables 2–5 show that penicillins have been chromatographically resolved on agarose and agar [65,84,85], silica gel plates [7,12,65,75,76], bonded methylsilicone fused-silica capillary columns [80], and reversed-phase columns [48,72,74,77–79,81,83].

TABLE 6

RECOVERY OF PENICILLINS G AND V FROM DIFFERENT SOLID-PHASE EXTRACTION UNITS

Four replicate analyses were conducted with each type of cartridge. Regular Sep-Pak and Sep-Pak plus are produced by Waters Chromatography (Mississauga, Canada). Bond Elut is produced by Varian (Harbor City, CA, USA). SPE* cartridges are distributed by Scientific Products and Equipment (Concord, Canada).

Solid-phase extraction unit	Detector response (mean ± S.D.) (mm)	
	Penicillin G	Penicillin V
$\geqslant 17\%$ Carbon loading		
t-C_{18} (Sep-Pak plus)	196.5 ± 3.4	92.1 ± 1.1
Bond Elut	171.8 ± 2.6	89.2 ± 1.3
SPE*	167.1 ± 2.8	84.5 ± 1.4
$\leqslant 14\%$ Carbon loading		
C_{18} (regular Sep-Pak)	147.2 ± 3.9	58.8 ± 2.4
SPE*	134.3 ± 2.8	57.5 ± 2.5

4.1. Thin-layer chromatographic method for penicillin residues

Ampicillin [65], procaine penicillin G [48,65,75], sodium penicillin G [7,76] and penicillin V [76] residues in animal tissues have all been analyzed by TLC. The most widely used stationary phase was commercially available silica gel or cellulose plates. Except for DePaolis *et al.* [48] who used autoradiochromatography for detection and a scintillation counter for quantitation, all the other TLC methods have used microbiological inhibition methods (bioautography) for detection and quantitation.

TLC with bioautographic detection has been used as a laboratory technique for the screening of veterinary drugs, including penicillins. The major drawbacks of this technique are that it is labor-intensive, costly, time-consuming, does not distinguish among the various penicillins, and is less sensitive than the screening tests used at the abattoirs for penicillin detection. For a regulatory laboratory without access to sophisticated instrumentation, this technique will generally detect penicillins above a 50-ng/g tolerance level and provide some measure of analytical support for a regulatory program.

4.2. Gas chromatographic methods for penicillin residues

Meetschen and Petz [80] (Table 3) have published the only GC method for the analysis of penicillins (penicillins G and V, methicillin, oxacillin, cloxacillin, dicloxacillin and nafcillin) in animal tissue. The penicillins, after isolation from animal tissue, were methylated, cleaned up further on diol cartridges, evaporated, and dissolved in cyclohexane containing two external standards, 2,4,6-triphenoxy-1,3,5-triazine and 2-(4-biphenyl)-5-(4-*tert.*-butylphenyl)-1,3,4-oxadiazole. This derivatization step was required to form not only the volatile methyl esters of the penicillins, but also to improve their chromatographic properties (thermal stability and decreased polarity) on the fused-silica capillary column. The use of a temperature-programmed vaporization injector instead of a conventional GC (on-column or flash vaporization) injector by the authors results in a markedly improved chromatographic sensitivity and reproducibility for the derivatized penicillins. Since the method involves extensive sample preparation steps with recoveries ranging from 48 to 83%, outside the range specified in section 3.1., it would have been more practical to have included internal standards added to the fortified samples just prior to extraction to correct for sample losses during sample preparation. The method would not be expected to work for the aminopenicillins, unless the amino groups are also derivatized. However, the detection limits of < 2 ng/g claimed for all the penicillins determined by this method make the procedure very attractive for regulatory use.

4.3. Liquid chromatographic methods for penicillin residues

The separation of penicillins from one another and from other endogenous or exogenous components in the tissue extracts has been achieved on octadecylsilyl-bonded reversed-phase HPLC columns [48,72,74,77–79,81,83] and eluted with buffers (at pH values ranging from 2 to 7) containing organic modifiers (methanol, acetonitrile). In the absence of ion-pair reagents, separation on the reversed-phase column occurs as a result of the partition between the non-polar stationary phase and the polar mobile phase; the most polar components elute first and the least polar components elute last.

Most penicillins do not have a specific UV chromophore. Even where absorption bands usually attributable to simple aromatic moieties exist (200–235 nm), these are generally not specific or intense. Greater sensitivity is normally achieved at wavelengths below 200 nm. However, at these wavelengths selectivity is poor, resulting in high background interference when trace analyses of tissue extracts are required. Penicillins are therefore chromatographed either in their underivatized (native) forms or as penicillin derivatives with improved UV chromophores.

4.3.1. Detection of native penicillins

4.3.1.1. UV detection. Moats [83], Terada *et al.* [78] and Nagata and Saeki [74] all detected penicillins separated on C_{18} reversed-phase columns by UV detection between 200 and 235 nm. Detection limits for penicillin G [78,83], cloxacillin [83] and

ampicillin [74] residues in animal tissues were determined to be 50, 50 and 30 ng/g, respectively. Under these conditions, the free bases (procaine or benzathine) formed when procaine or benzathine penicillin G salts dissolve in the tissue fluids may be detectable if they are extracted in the initial sample preparation steps. The chromatograms obtained by Terada *et al.* [78] and Nagata and Saeki [74] are less complex than that obtained by Moats [83] (see Figs. 2–4).

4.3.1.2. Mass spectrometry. Tyczkowska and coworkers [77,81] and Boison *et al.* [79] have all used the mass spectrometer as a specific detector for the detection of underivatized penicillins after they have been chromatographed on reversed-phase columns. Tyczkowska *et al.* [77] used thermospray LC–MS to confirm ampicillin, penicillin G and cloxacillin residues in spiked tissues at concentration levels of 10 ng/g, whereas Boison *et al.* [79] were able to confirm penicillin G in tissues at 5 ng/g. Full-scan mass spectra of the components eluting from the LC column into the thermospray ion source of the mass spectrometer, together with retention parameters on the LC column, provide both the qualitative and quantitative information needed to unambiguously identify and determine the penicillin(s) present in the animal tissue. These methods are of significant importance to regulatory agencies because identifications based on these methods can be used in support of regulatory action.

4.3.2. Detection of derivatized penicillins

4.3.2.1. UV detection. Only DePaolis *et al.* [48] and Boison *et al.* [72] have applied a pre-column derivatization method for the analysis of penicillins in animal tissues by HPLC. DePaolis *et al.* [48] subjected penicillin G in animal tissues to different cooking conditions and examined the degradation products [benzylpenicilloic, benzylpenilloic, benzylpenillic acids and α-(1-carboxyethyl)hydrogen benzylpenicilloate], formed in the process by HPLC (as their methyl esters) with UV detection at 254 nm. Boison *et al.* [72] formed the mercury mercaptide derivatives of penicillins G and V and detected them at 325 nm where very few endogenous components would absorb (see Fig. 5). In addition, the derivatization method used by Boison *et al.* [72], is specific only to compounds with an intact β-lactam ring.

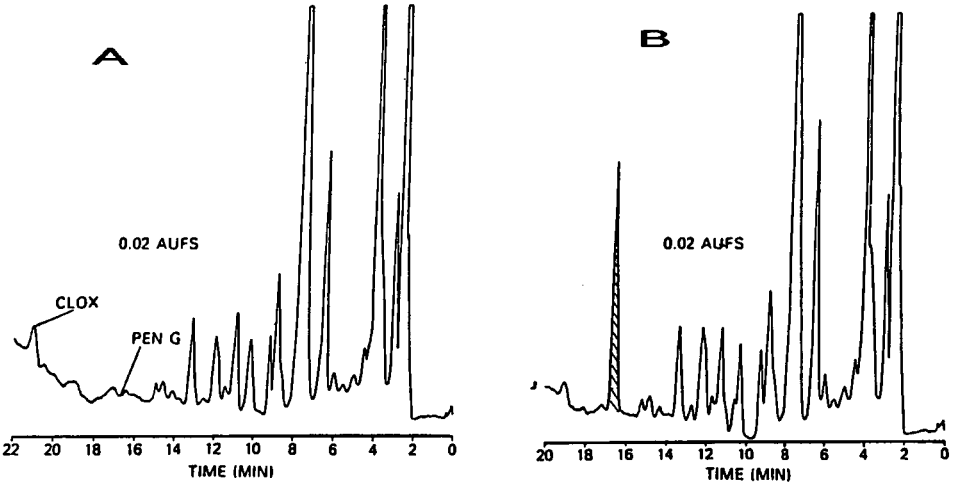


Fig. 2. Chromatograms of (A) a blank beef muscle extract after ammonium sulphate–acetonitrile clean-up, 0.2 g equivalent injected in 200 μl; (B) beef muscle spiked with 1 ppm sodium penicillin G. Varian Model 5000 liquid chromatograph, UV absorbance detector set at 220 nm, 0.02 a.u.f.s., Varian Micropak MCH-10 column, 30 cm × 4.6 mm I.D., solvent gradient 0.01 *M* phosphoric acid–acetonitrile (from 80:20 to 40:60 in 20 min). Flow-rate, 1 ml/min. Arrows indicate retention times of penicillin G and cloxacillin. A small interfering peak has a retention time near that of cloxacillin. The penicillin G peak in B is shaded. *y*-Axis represents the response in absorbance units and the *x*-axis represents the retention time. From ref. 83.

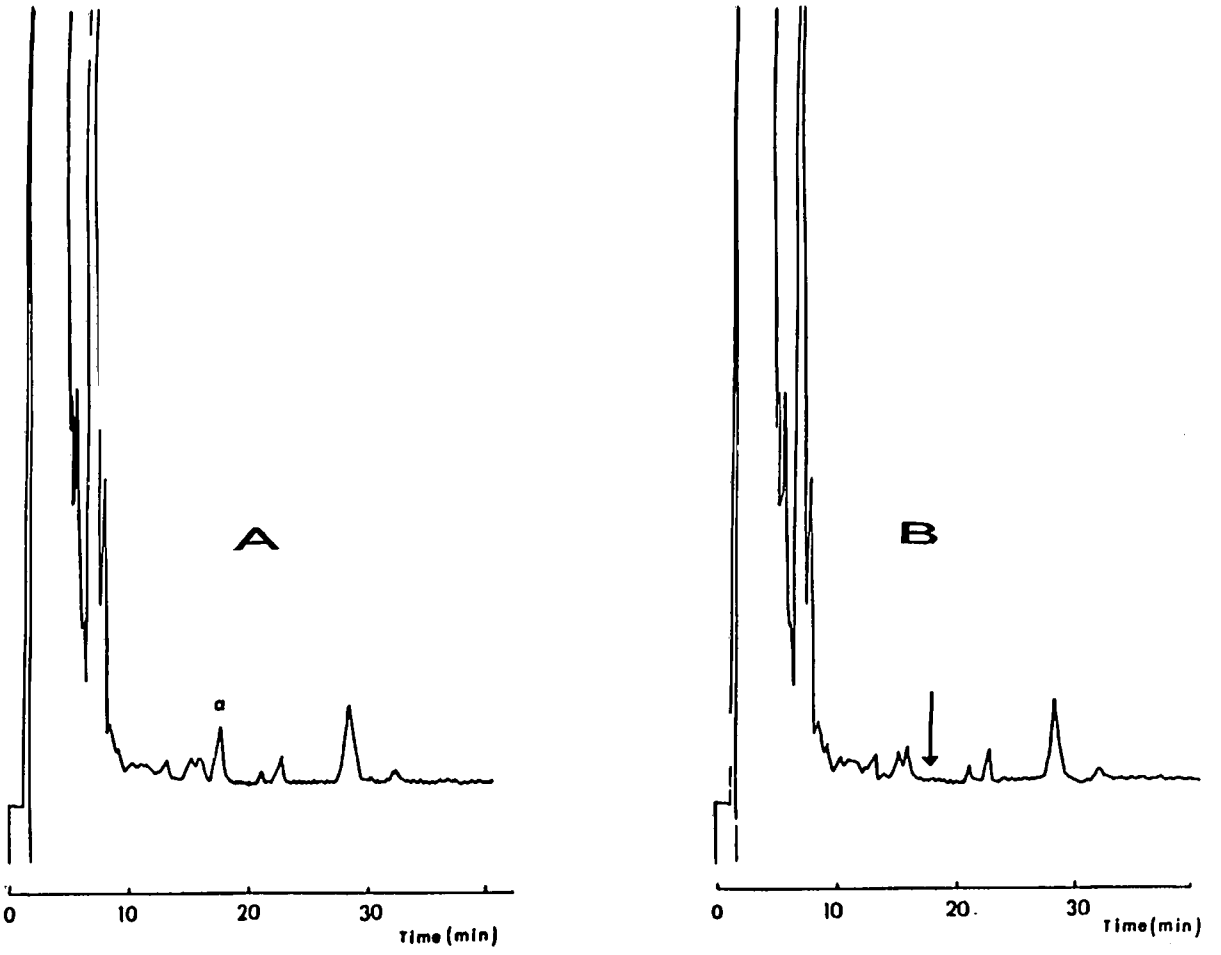


Fig. 3. Typical chromatogram of spiked and unspiked commercial yellow tail fish tissues: (A) 2 μg of ampicillin in 10 g of tissue (peak a = 20 ng ampicillin); (B) unspiked fish tissue (arrow indicates retention time of ampicillin). *y*-Axis represents absorbance and *x*-axis represents the retention time. From ref. 74.

This method is suitable for all the penicillins listed in Fig. 1, including the aminopenicillins, but will not detect free procaine or benzathine bases because they cannot form the mercury mercaptide penicillenic acid complexes. The mechanism for the formation of the mercury mercaptide penicillenic acid derivatives in the presence of 1,2,4-triazole as postulated by Haginaka *et al.* [86] is shown in Figs. 6 and 7.

4.3.2.2. Mass spectrometry. DePaolis *et al.* [48] used the direct insertion probe technique to obtain electron-impact (EI) and chemical ionization (CI) mass spectra in order to characterize some of the degradation products obtained after cooking penicillin in beef and chicken under various cooking conditions. For example, one of the degradation products after treatment with diazomethane was identified from the EI mass spectrum [*m/z* 452 (5.3%), 418 (9%), 393 (1%), 378 (1.8%), 359 (6.2%), 333 (6.7%), 317 (4.8%), 314 (5.7%), 291 (11.4%), 279 (13.3%), 255 (8.6%), 215 (11.4%), 188 (18%), 174 (100%), 118 (30%), 114 (41%), 91 (63%)] and CI mass spectrum (gave an M + 1 ion at *m/z* 453) as α-[(1-methoxycarbonyl)ethyl]-4-methyl

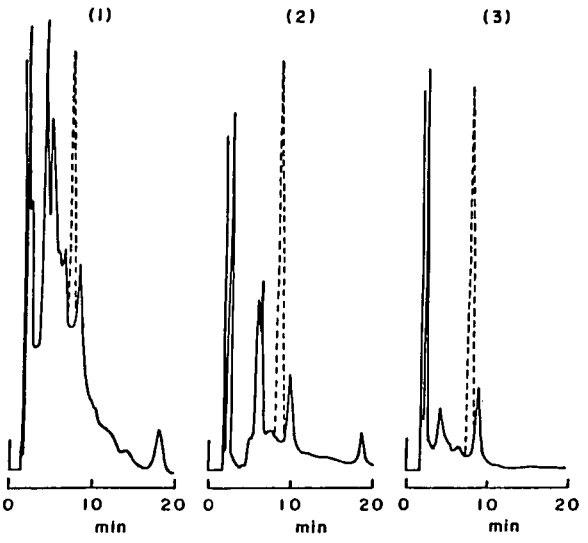


Fig. 4. Typical chromatograms obtained from (1) cattle liver, (2) kidney and (3) muscle by the overall procedure. Broken line: sample spiked with 1.0 μg/g sodium penicillin G. *y*-Axis represents UV response and *x*-axis represents the retention time. From ref. 78.

benzylpenicilloate. This characterization was confirmed by the synthesis of α-(1-carboxyethyl)hydrogen benzylpenicilloate and treatment with diazomethane to form the methylated product which was found to have identical mass spectral characteristics.

4.3.2.3. Nuclear magnetic resonance (NMR) spectroscopy. DePaolis *et al.* [48] also used NMR spectra to support the identification and characterization of some of the isolated degradation products (benzylpenicilloic acid) obtained from penicillin in tissues after cooking. Since then no one has pursued this method of detection primarily because microgram quantities (compared with nanogram amounts) of purified analytes are required for the analysis to be meaningful.

4.4 Electrophoretic methods for penicillin residues in animal tissues

Table 6 shows the pioneering work done by Smither and co-workers [65,84,85] in the application of the slab zone electrophoresis technique for penicillin analysis in animal tissues. With this technique, amoxicillin, ampicillin, penicillin G, penicillin V, methicillin, phenethicillin and cloxacillin in tissue extracts were separated from one another, identified and quantified. In my opinion, the slower slab zone technique has given way to newer refined techniques such as capillary zone electrophoresis and micellar electrokinetic capillary chromatography which appear to offer better sensitivity for the analysis of penicillins and ease of operation. Since higher potential gradients can be achieved with the newer techniques, the time for analysis, which is usually hours for the slab technique, can be substantially reduced to a few minutes [87,88].

5. APPLICATIONS TO TISSUE RESIDUE STUDIES

At a recent meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [89], it was recommended that a maximum residue limit (MRL) for total residues of benzylpenicillin of 0.05 mg/kg for kidney, liver and muscle be established for all food-animal species. This recommendation has been adopted, at least in part, by a number of countries, including USA and Canada. In Canada, for example, the prescribed tolerances are 0.05 mg/kg for penicillin G in cattle, 0.01 mg/kg in turkeys and zero in swine [90]. Similar tolerances have been set in the USA and in other countries. An MRL is the maximum concentration (mg/kg body mass) of residue resulting from the use of a veterinary drug that is recommended by the Codex Alimentarius Commision to be legally permitted as acceptable in food. The JECFA recommendation implies that analytical methods used by regulatory agencies to monitor benzylpenicillin residues in food-animal tissues must be able to detect and/or confirm benzylpenicillin residues at levels of 0.05 mg/kg. It was also noted at this meeting that there was only one published penicillin G tissue residue depletion study [55] based on a chromatographic method of analysis. In that study, tissue residues remaining in kidney, liver and non-injection site muscle of fifteen cross-bred pigs injected intramuscularly with 13 200 I.U. of procaine penicillin G per kg body weight and slaughtered in groups of three at 4 h and 1, 2, 4 and 8 days after the injection were determined by HPLC. In the research laboratory at the Health of Animals Laboratory, extensive tissue depletion studies in feedlot steers have been recently conducted by Korsrud and co-workers [91,92] using the

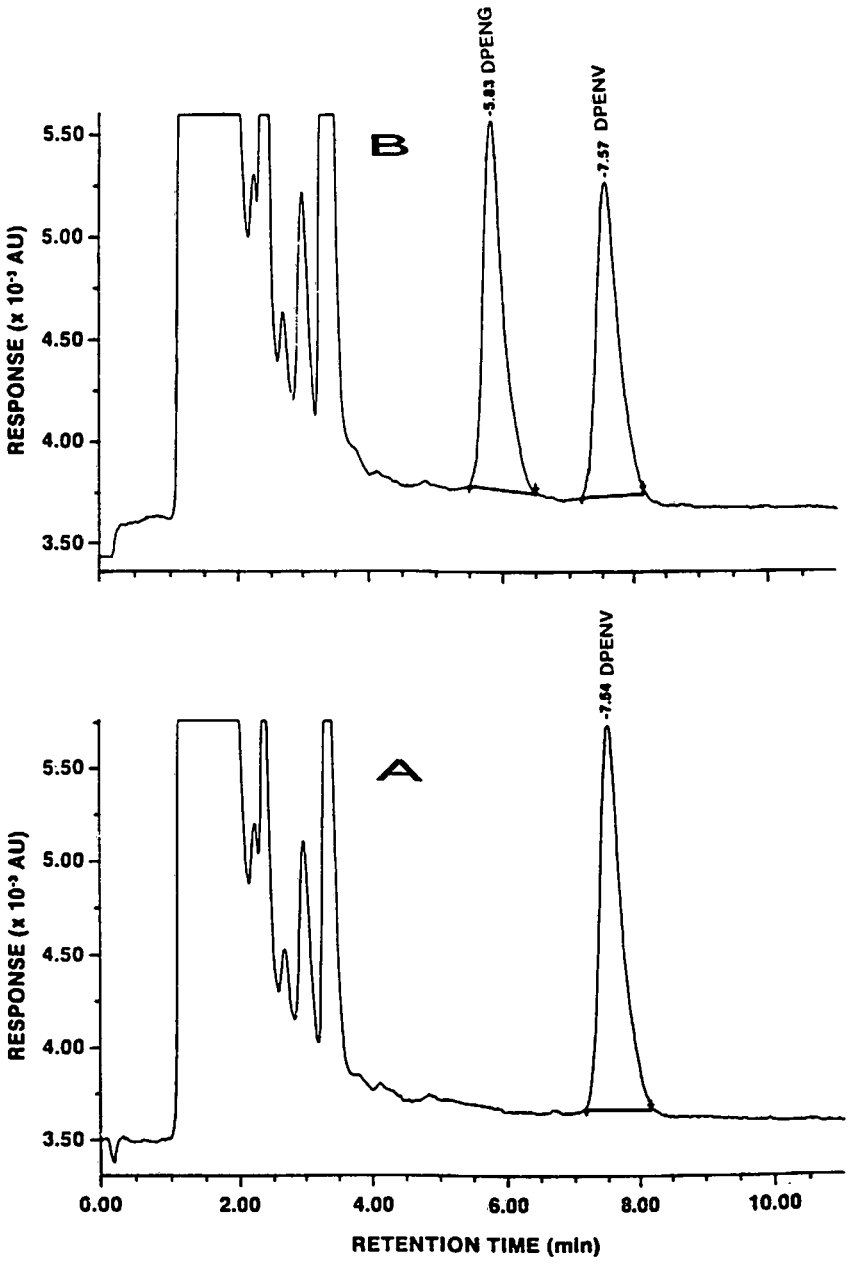


Fig. 5. Chromatogram of a 50-μl injection of a derivatized extract from a control muscle tissue. (A) A control muscle tissue fortified with procaine penicillin G at 200 I.U./kg; (B) a constant amount of penicillin V (300 μg/kg). Peaks represent the mercury mercaptide complexes of penicillin G (DPENG) and penicillin V (DPENV) detected at 325 nm at 0.005 a.u.f.s. From ref. 72.

method developed by Boison *et al.* [72] to measure tissue concentrations of penicillin G when extra-label doses of procaine penicillin G, either alone or in combination with its long-acting analogue, benzathine penicillin G, were injected intramuscularly or subcutaneously to steers. In these studies, the

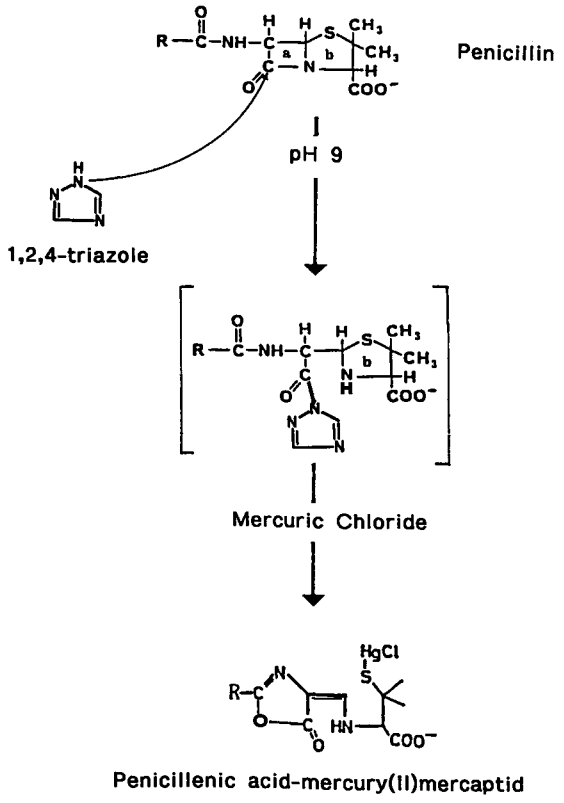


Fig. 6. Postulated mechanism for the 1,2,4-triazole base-catalysed formation of penicillenic acid mercury mercaptides [86].

depletion of benzylpenicillin from non-injection site muscles, kidneys, livers and diaphragm from steers slaughtered 1, 2, 3, 4, 8 and 12 days after the administration of penicillin formulations at dosages ranging from the label dose to 8.2 times the label dose were followed by HPLC. Results from these studies are being used to establish withdrawal periods required when penicillin G formulations are used in an extra-label manner in cattle in Canada [93]. Boison *et al.* [94] have also used the HPLC method to determine the effect of cold-temperature (−20°C and −76°C) storage on the stability of benzylpenicillin residues in plasma and tissues of food-producing animals. The authors found that significant losses of penicillin G residues in gluteal muscle stored at −20°C occurred in as little as 10 days of storage. Such samples lost about 50% of their initial concentration of penicillin G after only 10 days of storage. Losses were found to be less, but still significant, for penicillin-incurred plasma stored at −20°C for more than 2 months before they were analyzed by HPLC. However, penicillin G residues in tissues stored at −76°C were found to be very stable. It was therefore recommended that analytical laboratories receiving samples for penicillin analysis should take note of the extent to which penicillins deplete from tissues and biological fluids and modify their analytical and sample storage protocols ac-

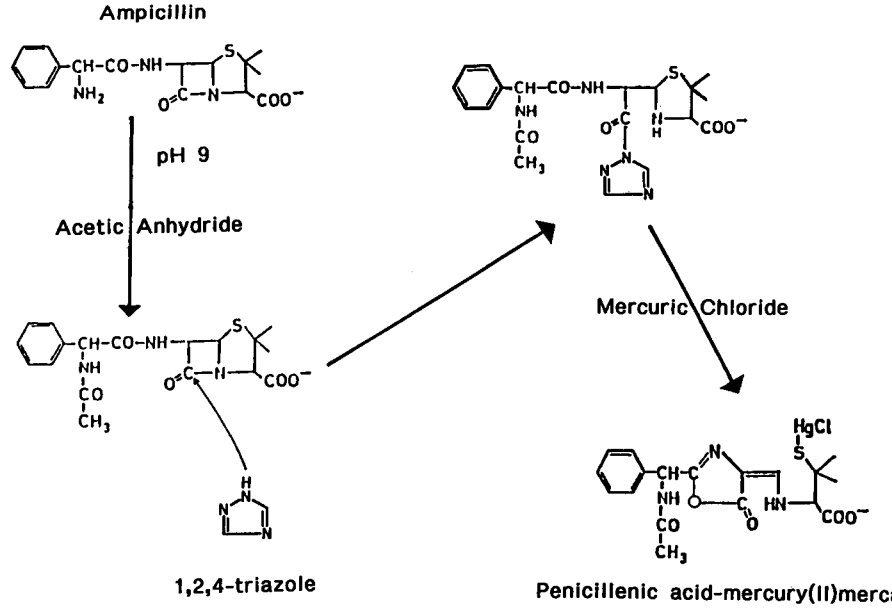


Fig. 7. Postulated mechanism for the 1,2,4-triazole base-catalysed reaction of aminopenicillins with mercuric chloride to form the N-acetylpenicillenic acid mercury mercaptide [86].

cordingly. Otherwise. quantitative analyses conducted on such stored samples will reflect residue concentrations at the time of analysis, but may not adequately reflect concentrations at time of submission.

6. FUTURE PROSPECTS

Of the β-lactam antibiotics commonly used for the treatment of food-producing animals, benzylpenicillin (penicillin G) is the drug for which most of the chromatographic methods of analysis have been developed. The emphasis placed on the development of chromatographic methods of analysis for penicillin residues in animal tissues is probably the direct result of the observations made in several survey studies conducted to monitor the prevalence and incidence of veterinary drug residues in animal tissues [7–10]. While the development of methods for penicillin G has now reached a point where penicillin residues in animal tissues can be adequately determined down to concentrations of 5 ng/g, researchers involved in the development of chemical methods for the analysis of other β-lactam antibiotics listed in Fig. 1 should be encouraged to pursue method developments for these other drugs more vigorously, even though these other veterinary drugs appear to be used less frequently in livestock production.

There is no doubt that if producers begin to use these other penicillins more frequently in animal production than benzylpenicillin, the screening tests described in section 2 and used at most abattoirs will detect them. However, it may not be possible, in the absence of sensitive quantitative methods, for the regulatory laboratory to confirm the identity and the concentration of the drug present to support regulatory action. In anticipation of the possible use of these less commonly used penicillins by producers in the future, Boison *et al.* [95], have modified the isocratic LC method [72] developed for penicillin G to a gradient mode to allow the separation and determination of ampicillin, amoxicillin, penicillin V, penicillin G and cloxacillin from tissue extracts (see Fig. 8). This method will soon be evaluated using

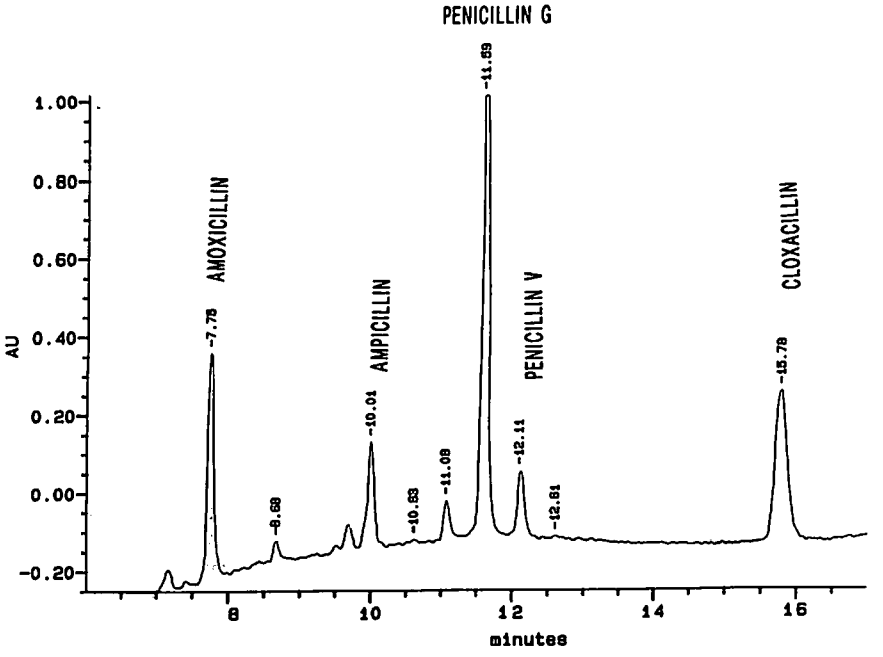


Fig. 8. Gradient elution of five penicillins (mercaptide derivatives of amoxicillin, ampicillin; penicillin G, penicillin V and cloxacillin) extracted from bovine muscle using the method in ref. 72 and separated on a Nova-Pak C_{18} column (15 cm × 4.6 mm I.D.; 5 μm Waters Chromatography) at a flow-rate of 0.8 ml/min with UV detection at 325 nm (0.005 a.u.f.s.). Gradient conditions were as follows: 0–1.4 min, 10:90 A–B; 2–5 min, 20:80 A–B; 5–12 min, 30:70 A–B; 12–14 min, 60:40 A–B; 14–16 min, 60:40 A–B and 16–17 min, 10:90 A–B; A = acetonitrile; B = 0.1 *M* phosphate buffer pH = 6.5 containing 0.0157 *M* sodium thiosulphate.

incurred tissues obtained from animals treated with the other β-lactams, after which it could be incorporated into the laboratory protocol for a multi-residue β-lactam method.

One aspect of the development of methods for detecting penicillin residues in animal tissues which could progress substantially in the near future is in the sample preparation area, where techniques that require little or no sample preparation at all will be developed and used more frequently. These techniques will include capillary electrophoresis and micellar electrokinetic capillary chromatography (MECC). High-performance capillary zone electrophoresis (HPCZE) is performed in a fused-silica capillary column, 20–100 cm × 25–100 μm I.D., filled with an appropriate buffer. Sample (1–30 nl) is introduced into the capillary and the opposite ends of the capillary and electrodes are immersed in small buffer-containing reservoirs. Electric potentials ranging from 200 to 300 V/cm are then applied to the electrodes and charged components in the sample end of the capillary migrate in very narrow bands through the capillary to the electrode of opposite polarity. Separation is based on differences in the electrophoretic mobilities of the charged components. This technique has already been applied to the separation and analysis of several β-lactam antibiotics. For example, Yeo *et al.* [96] used HPCZE to separate penicillin G, ampicillin, amoxicillin, chlortetracycline, nystatin and tylosin tartrate in methanol and ampicillin in a commercial tablet. The major drawback with classical CZE is that it deals with aqueous electrolytic systems, and components can only be separated if they are charged and soluble in water. A variant of this technique, referred to as MECC, combines the advantage of CZE in the separation of charged species with the selectivity required for the separation of uncharged compounds. In MECC, both charged and uncharged compounds, and compounds that are almost insoluble in water, can be separated, because of the hydrophobic nature of the micelles. This method is based on micellar solubilization and electrophoretic migration of the micelle. Solutes are separated by their differential distribution between the micelle and the surrounding aqueous phase and the differential migration of the two phases. For example, Rahn [87] has used MECC to separate the nine β-lactam antibiotics (amoxicillin, ampicillin, 6-aminopenicillanic acid, oxacillin, cloxacillin, ticarcillin, nafcillin and dicloxacillin) in 15 min (see Fig. 9). Nishi *et al.* [88] have applied the same technique to separate seven penicillins (benzylpenicillin, ampicillin, carbenicillin, sulbenicillin, piperacillin, aspoxicillin and amoxicillin) and nine cephalosporin antibiotics in under 20 min (see Figs. 10 and 11). These methods, admittedly, have been applied to standard solutions, but their power to resolve compounds (charged and neutrals) should present obvious advantages in research for developing methods for penicillin residues with a wide range of polarities and solubilities in aqueous and non-aqueous solvents.

Immunoaffinity (antibody-mediated) clean-up methods are becoming popular for the analysis of veterinary drug residues [97–101]. The technique is based on a specific binding between a hapten and an antibody (covalently bound to an activated support) raised against it. In this technique, a sample solution is applied to the immunoaffinity column, and the immobilized antibodies react immunochemically with the specific drug molecules to retain them while the matrix components pass to waste. The column is then washed and the retained components of interest are eluted and analyzed or cleaned up further for analysis. This technique has been used as a clean-up and concentration step prior to chromatographic analysis of chloramphenicol in milk and eggs [97] and tissues [98]. Monoclonal antibodies are highly

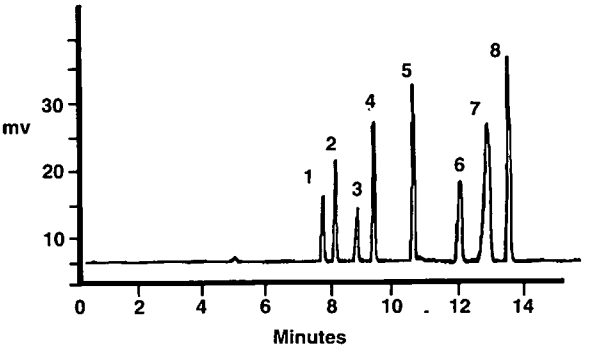


Fig. 9. Micellar electrokinetic capillary chromatography of benzylpenicillin and related antibiotics with UV detection at 214 nm. Peaks: 1 = amoxicillin; 2 = ampicillin; 3 = 6-aminopenicillanic acid; 4 = oxacillin; 5 = cloxacillin; 6 = ticarcillin; 7 = nafcillin; 8 = dicloxacillin. From ref. 87.

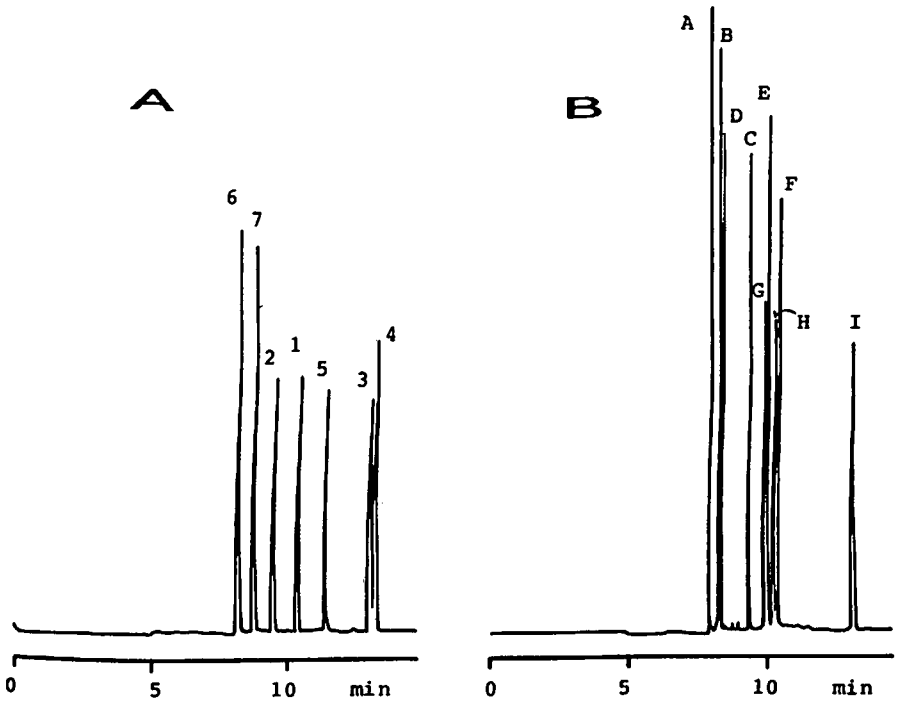


Fig. 10. Micellar electrokinetic capillary chromatography with sodium dodecyl sulphate (SDS) of seven penicillins (A) and nine cephalosporins (B) at 20 kV d.c. voltage with detection at 210 or 220 nm. Peaks: 1 = benzylpenicillin; 2 = ampicillin; 3 = carbenicillin; 4 = sulbenicillin; 5 = piperacillin; 6 = aspoxicillin; 7 = amoxicillin; A = ceftazidime; B = cefotaxime; C = cefoperazone; D = cefmenoxime; E = cefpiramide; F = ceftriaxone; G = cefpimizole; H = cefminox; I = C-TA. *y*-Axis represents UV response and *x*-axis the retention time. From ref. 87.

specific. Therefore, a large amount of sample can be subjected to antibody-mediated clean-up without retention of the matrix components, thus enabling the determination of very low levels of drugs of interest. However, because immunological reactions occur under physiological conditions, only aqueous solutions, and therefore water-soluble compounds, can be submitted to this form of sample clean-up.

The formation of chemical derivatives of penicillins with suitable chromophores to enhance detection sensitivities by HPLC analysis will be another area that will see significant improvement in the future. Several authors have demonstrated that fluoro-labelled penicillin derivatives [16,36,102–106], colored penicillin complexes [107–109] and penicillin derivatives with UV-enhanced chromophores [27,72,78,110–114] can be used to detect low levels of penicillins in pharmaceutical preparations [105–107] and biological fluids, including urine [27,111,112], milk [36], plasma [27,111,112] and fermentation broths [104,109]. Fluoro complexes, including dansylaziridine [104], fluorescamine [105] and *o*-phthaldialdehyde [106] derivatives, have been used in pre- or post-column HPLC methods to determine the concentrations of penicillins in biological fluids and fermentation media down to sub-ng/g levels but this approach has not been used for the analysis of penicillins in animal tissues. Except in the case of post-column reaction techniques where there is increased capital expense because of the cost of commercial post-column reactors, there should be no reason why these techniques cannot be applied to tissue analysis to improve the detection limits of the assays.

Confirmation of penicillins in animal tissues by MS techniques is another area where a lot of activity will take place. A lot of fundamental studies have already been done on the fragmentation and characterization of mass spectra of penicillins [77,79,81,82, 115–124]. While a few of these studies have been

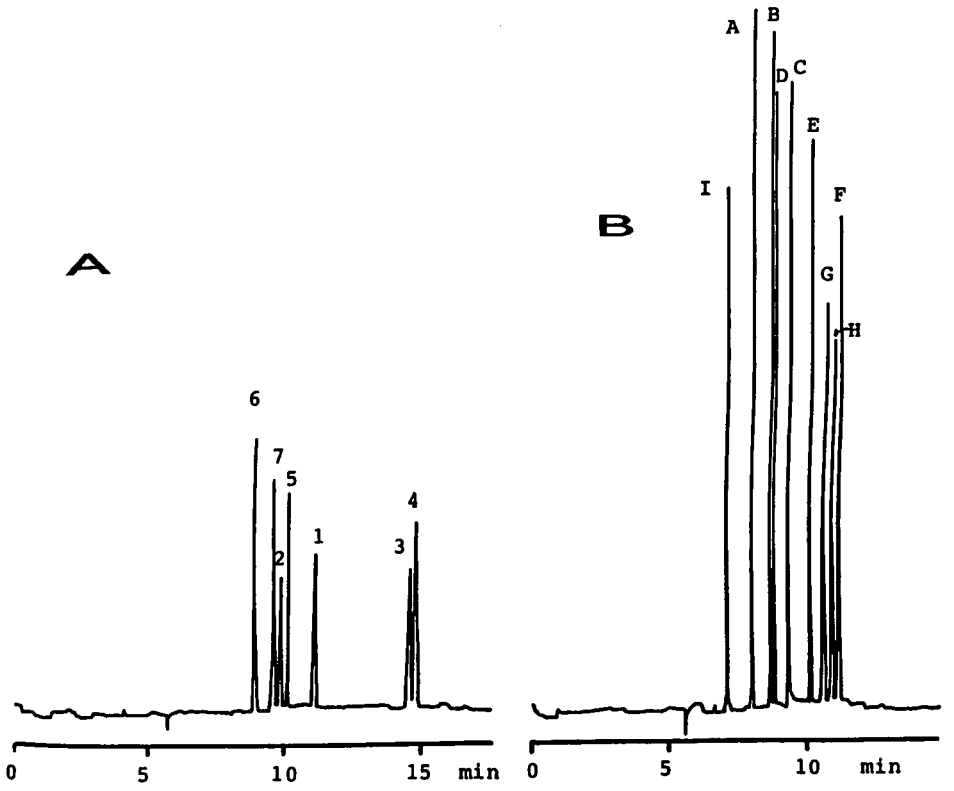


Fig. 11. Micellar electrokinetic capillary chromatography with N-lauroyl-N-methyltaurate of seven penicillins (A) and nine cephalosporins (B) at 20 kV d.c. voltage with detection at 210 or 220 nm. Peak identification as in Fig. 10 [88]. *y*-Axis represents UV detector response and *x*-axis represents the retention time.

conducted on penicillins in tissue matrices [77,79,82, 118], most of the MS work has been limited to standard solutions of the pure compounds [81,115–117,119–123] or biological fluids [124]. New developments in interfacing mass spectrometers [125,126] with LC instruments (plasmaspray, thermospray, ionspray and moving belt) are making it easier to simultaneously determine and provide confirmation of penicillins. Since the cost of a mass spectrometer is prohibitive to most institutions, the new developments in interface technology coupled with improvements in vacuum pump technology will, in the future, enable LC equipment to be coupled directly to simple, inexpensive bench-top mass spectrometers [127]. This will enable affordable mass spectrometers with LC interfaces to be available on the market as we see today with GC–MS instruments.

Finally, one other area in regulatory laboratories where improvement will take place is in the area of laboratory automation for the analysis of penicillin residues in animal tissues. Most methods developed to date require analyst involvement from the initial tissue extraction stages through to the end of the sample preparation stage when the samples are loaded into an HPLC or GC system equipped with an automated sampling unit. These methods become heavily resource-based. As part of a research endeavor to introduce some measure of automated sample analysis into the diagnostic program at the Health of Animals Laboratory, a commercially available sample preparation unit has recently been acquired for evaluation and to determine whether it can be adapted to perform the procedures described in the method published by Boison *et al.* [72]. The sample preparation unit is claimed to be capable of unattended conditioning of solid-phase extraction

cartridges, loading available sample extracts onto the conditioned solid-phase extraction cartridges, eluting them with defined solvents and even adding chemical reagents to the eluent held in controlled-temperature baths for reaction and automatically injecting the sample into an HPLC system. According to the method developed by Boison *et al.* [72] for the determination of penicillin G in animal tissues, all that the analyst has to do is spend 20–30 min per day homogenizing the tissue samples and extracting them for the sample preparation unit to complete the analyses. To make the operation of the automated sample unit practical for diagnostic use, however, the volume of tissue extract obtained according to the method by Boison *et al.* [72] has to be reduced substantially to meet volume constraints imposed by the automated sample preparation unit. This is the subject of current research in the research laboratory at the Health of Animals Laboratory, Saskatoon, Canada.

7. CONCLUSIONS

Very few chromatographic methods (TLC, GC, HPLC and electrophoresis) have been developed and applied to the analysis of penicillin residues in animal tissues. There is, however, a need to develop such methods for the determination of penicillin residues because screening methods used in the field as a prelude to laboratory analysis have better sensitivities than most of the laboratory methods. When sensitive laboratory methods become available for all the penicillins, regulatory agencies can use the data generated from depletion studies of these veterinary drugs in animal tissues to define withdrawal periods and establish tolerances and action levels for the use of all the β-lactam antibiotics in animal tissues. The definition of withdrawal periods using sensitive, quantitative laboratory methods when these drugs are used in an extra-label manner will be extremely useful, because it will provide the much needed guidelines required to encourage responsible agricultural practice among livestock producers and thereby reduce the incidence of β-lactam antibiotic residues in food-producing animals and improve the global livestock trade. Emerging techniques, such as CZE, MECC and affinity chromatography will, in the future, be applied extensively to the analysis of penicillin residues in food animal tissues. As well, laboratory automation of analytical procedures for the analysis of penicillin residues in animal tissues will be incorporated into diagnostic laboratory protocols and LC–MS confimatory methods will continue to play an important role in regulatory analysis.

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Review

Chromatographic methods for tetracycline analysis in foods

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ABSTRACT

The tetracyclines have served for decades as an important class of antibiotics in food animal health and production. As such, they have also been a source of concern for residue monitoring authorities around the world. In response to this concern a number of microbial inhibition, immunoassay and bacterial receptor methods have evolved for the detection of this class of compounds in various foods of animal origin. However, these methods often lack specificity and are subject to false positive and false negative results. For these reasons a number of chromatographic methods for the separation and determination of the tetracyclines isolated from foods have been developed that are capable of identifying and quantifying individual tetracycline drugs. We present here an overview of tetracycline analytical methods, including microbial inhibition, immunoassay and receptor technologies for detection, techniques for isolation from food matrices, and thin-layer chromatographic, high-performance liquid chromatographic, gas chromatographic and mass spectrometric procedures for determination of this class of compounds. A discussion of the variables involved in such methodology and a review of method criteria are offered.

CONTENTS

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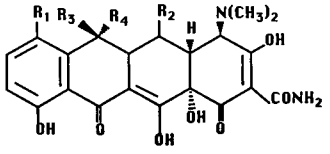
1. INTRODUCTION

Over the last two decades there has been an increased use and availability of veterinary therapeutic agents to maintain the health of and increase the production from food animals. This increased usage has been paralleled by a rising concern regarding the presence of residues from such agents in the food supply. The presence of drug residues is often due to the improper observance of drug withdrawal times and is most commonly seen, in the USA, for the various classes of antibiotic drugs [1]. The tetracycline antibiotics are one of these classes and have long been a source of concern to residue monitoring programs.

The Food Safety and Inspection Service (FSIS) branch of the United States Department of Agriculture (USDA) has included the antibiotics tetracycline (TC), chlortetracycline (CTC) and oxytetracycline (OTC; Fig. 1, a–c, respectively) in their "Compound Evaluation and Analytical Capability National Residue Program Plan" [2] for many years and routinely monitor for the presence of these compounds in tissues from food animals. Internationally, these compounds, as well as the compounds doxycycline (DC; 1d, Fig. 1) and minocycline (MC; 1e, Fig. 1) and others, are also of concern as residues in animal derived foods. The governmental tolerances and/or action levels for these compounds vary among countries and among animal species, based on differing predicted consumption rates and safety factors. The FSIS, for example, utilizes levels ranging from zero tolerance to 4 μg/g for monitoring the various species and tissues examined [2]. Monitoring for these compounds in the various species is conducted, in most cases, by a Swab test or other microbiological assay [2] utilizing transudate or homogenate from kidney, liver or muscle tissues. Preliminary identification of the particular residue involved can be based on thin-layer chromatographic analysis of the tissue extract after the conversion of the component tetracycline to its anhydro derivative [2,3]. More exacting determinative and confirmatory methodology is then applied to those samples suspected of being in violation.



a. Tetracycline, R_1=R_2= H, R_3= OH, R_4= CH_3
b. Chlortetracycline, R_1=Cl, R_2= H, R_3= OH, R_4= CH_3
c. Oxytetracycline, R_1= H, R_2=R_3= OH, R_4= CH_3
d. Doxycycline, R_1=R_3= H, R_2= OH, R_4= CH_3
e. Minocycline, R_1= $N(CH_3)_2$, R_2=R_3=R_4= H

Fig. 1. Structures of the various tetracyclines.

In this review we will focus on the latter aspect of tetracycline analyses, examining the chromatographic techniques that have been successfully applied to the isolation and determination of these compounds as they occur as residues in food animal matrices. A number of reviews concerning the analysis of tetracyclines in general or as residues in foods of animal origin appeared in the mid-1980s [4–8] and we will refer to these reviews *in toto* for some aspects of the matters addressed here. Further, given the importance of utilizing rapid screening technologies in food monitoring programs to determine to which samples these more elaborate determinative methods should be applied, we also provide an overview of the microbiological/immunoassay approaches presently available. From this information conclusions regarding the state of the art of tetracycline analysis and the directions for future investigation are drawn and offered for consideration.

2. MICROBIAL AND IMMUNOASSAY DETECTION OF TETRACYLINES

Tetracyclines have traditionally been detected in animal tissues and fluids by microbial inhibition tests (MIT). These MITs are in wide use today but a number of other detection methods have been de-

veloped in the last decade and are also available. These include the competitive bacterial receptor binding assay, enzyme immunoassay, and bioautography. At present such residue screening methods are not validated by any federal agency [9]. There are seven methods listed as AOAC Official Methods of Analysis for the detection of antibacterials in milk, but only one official method for the detection of tetracyclines [10]. No official methods are listed for the detection of tetracycline residues in animal tissues, however.

2.1. Microbial inhibition tests

All MITs are based on the inhibition of bacterial growth by residues of antibacterial compound(s) present in milk or tissue. Early assays for chlortetracycline and oxytetracycline residues in milk utilized the reduction of methylene blue as an indicator of bacterial growth [11,12]. Numerous MIT methods for the detection and quantitation of tetracycline have since been described [13–17]. MITs are non-specific and interferences from other antibacterials can occur. Selective sensitivity for tetracyclines or other antibiotics can be obtained by changes in the culture medium, indicator bacteria, or pH [18,19]. Microbial methods measure only the parent drug and microbiologically active metabolites [20]. Imprecision occurs as a result of zone size differences between plates. Zone size may vary as a result of differences in agar layer thickness, agar quality, uneven seeding of bacterial spores on the agar surface, or incubator temperature variation [14]. Plate assay MITs are performed by streaking a uniform suspension of indicator bacterial spores over an agar medium. Swabs or disks soaked in a body fluid are placed on the plate and incubated. A positive control is provided by a neomycin sensitivity disk. The observed end point can be a zone of inhibition surrounding a sample or a color change resulting from pH changes. MITs currently in use for screening tissues for tetracyclines by the USDA–FSIS are the Swab test on premises (STOP), live animal Swab test (LAST), and the calf antibiotic and sulfa test (CAST) [21]. The USDA–FSIS is currently evaluating the fast antibiotic screen test (FAST) as a replacement for the STOP and CAST methods [17].

2.2. Swab test on premises

The STOP is used to detect antibiotic residues in kidney and other tissues of slaughter animals [16]. The STOP method is relatively simple and requires only a few minutes [22]. A cotton swab is inserted directly into the meat sample, left in place for 30 min, and the cotton tip is placed on a test plate containing Difco antibiotic medium No. 5 previously streaked with a spore suspension of *Bacillus subtilis*. The plate is incubated at 29°C overnight (16–20 h) and observed for inhibition of bacterial growth surrounding the swab. Johnston *et al.* [16] reported 94% agreement with results of STOP and standard microbial assays. Korsrud and MacNeil [18] reported varying sensitivity with different media using standard solutions of tetracyclines. With the standard antibiotic medium No. 5, limits of detection (LODs, μg/ml) were 6.2 (CTC), 3.1 (OTC) and 1.6 (TC). With antibiotic medium No. 2, LODs were 0.06 (CTC) and 1.6 (OTC and TC). Minimal detectable levels using antibiotic medium No. 5 as reported by Johnston *et al.* [16], were 0.01 μg/ml (CTC) and 0.08 μg/ml for OTC and TC. In a comparison of STOP, HPLC, MIT and thin-layer chromatography–bioautography (TLC-B) by MacNeil *et al.* [23], STOP lacked the sensitivity of HPLC but had greater or lesser sensitivity for OTC than TLCB or MIT depending on the growth medium used.

2.3. Calf antibiotic and sulfa test

The CAST procedure was introduced by the USDA to increase sulfonamide detection sensitivity in bob veal calves but is also sensitive to a variety of other antimicrobials including tetracyclines. The degree of inhibition varies with the compound tested [24]. The CAST procedure is similiar to the STOP but uses Mueller–Hinton medium and *Bacillus megaterium* ATCC 9885 as the indicator bacteria, and is incubated at 44°C. Plates are read as for the STOP procedure and kidney is used as the sample tissue. Korsrud and MacNeil [18] reported the CAST procedure was more sensitive than the STOP procedure for standard solutions of 22 antibiotics tested including CTC, OTC and TC. Minimum detectable levels (μg/ml) were 0.2 (CTC), 0.8 (OTC) and 0.4 (TC).

2.4. Live animal swab test

The LAST procedure is a modification of the STOP procedure differing only in the amount of *B. subtilis* used [25]. It is used for preslaughter field screening of residues in urine and for prediction of residues in edible tissue. It was the first on-farm test available for screening live cattle for possible residues and is based on the correlation between urine and tissue residue levels. Urine or blood samples may be used [25,26]. Two sterile swabs are dipped in a urine sample and placed on the LAST plate containing antibiotic medium No. 5 streaked with *B. subtilis* ATCC 6633 spores . A neomycin disc is used as a positive control, and incubation and test interpretation are as for the STOP procedure. Several reports indicate a high incidence of false positive results using the LAST assay. In one study 75% (15 or 20) of untreated cows showed a positive result [27]. Tritschler *et al.* [25] reported 5.4% positive results and 19.9% questionable results from 221 untreated dairy cows and heifers. TerHune and Upson [28] had varying results for LAST detection of OTC when compared to standard quantitative OTC MIT procedures. LAST was 100% accurate when urine OTC concentration was >4.3 μg/ml and 60% accurate when urine OTC concentration was <4.3 μg/ml. Some 20% of LAST results were false positive and 20% were false negative. False positive results were associated with high urine osmolarity and high urine pH, apparently resulting in inhibition of bacterial growth. False negative samples were associated with dilute urine. In this study LAST was 100% accurate in detecting OTC in the urine and predicting tissue OTC residues when OTC concentration was at therapeutic levels. However, LAST did not detect OTC in the urine or predict OTC concentrations of 0.1–0.4 μg/g in tissue.

2.5. Fast antibiotic screen test

The FAST is a new procedure under evaluation by USDA–FSIS which provides results within 6 h. It has undergone field trials involving 10 000 samples for comparison with STOP and CAST for sensitivity. The FAST assay is similiar to the CAST procedure but the FAST growth medium contains sugar and a purple dye. Bacterial metabolism of the sugar results in acid production causing a color change from purple to yellow for the pH sensitive dye used. A sterile cotton swab is saturated with fluid from a tissue sample and placed on a plate of growth medium streaked with bacterial spores and incubated for 6 h. A purple zone surrounding the sample swab indicates the presence of antimicrobial agent(s) [17].

2.6. Delvotest P

This test is a qualitative color reaction test based on acid production by *Bacillus stearothermophilius* var. *calidolactis*. This changes the color of bromocreosol purple to yellow. If antibacterials are present, bacterial growth is inhibited and the purple color remains. Delvotest P is an AOAC Official Method for β-lactams in milk. Sensitivity for β-lactams is $\geqslant 0.005$ IU/ml milk. β-Lactam residue is confirmed using penicillinase [10]. It will also detect a wide range of antibiotics including TC at 0.2 μg/ml and OTC at 0.3 μg/ml [26]. Macaulay and Packard [29] reported 11% false positives with this test. Delvotest P is simple to run and the color change is easily evaluated as blue *vs.* yellow. A disadvantage is the 2.75-h analysis time.

2.7. Brilliant Black reduction test

The Brilliant Black reduction test is another qualitative color reaction test and can be used to detect antibiotic residues in milk and tissue. *Bacillus stearothermophilus* is the test organism used with an assay medium containing brilliant black indicator. The assay medium remains blue if bacterial growth is inhibited by antibiotic residues, but if no residues are present the growth of the bacteria reduces the indicator to a yellow color. Limit of detection of tissue extracts for OTC is 0.1 μg/ml [30].

2.8. Competitive receptor binding assay

Competitive receptor binding assay (Charm II test) is a competitive microbial receptor binding assay that can detect residues of seven classes of antibiotics. It is the only AOAC Official Method of Analysis for tetracyclines in milk [31]. Serum, urine, egg, honey and tisue extracts may also be used. In this method, microbial cells with specific receptor sites are added to milk or tissue extract containing added ^{3}H-labelled tetracycline. The [^{3}H]tetracycline competes with any residues of the tetracycline family present in the sample for the available bacterial receptors. Following centrifugation, the sample is decanted, the precipitate is resuspended, com-

bined with scintillation fluid and its activity measured using a scintillation counter. Sample activity is compared to a zero standard and the level of radioactivity is inversely related to the residue level of the sample. The level of radioactivity used ([^{3}H]tetracycline 0.5 μCi/umol, 0.052 μCi/test) is exempt from Nuclear Regulatory and Agreement State regulations [31]. Limits of detection (ng/ml) in milk are 3 (CTC), 6 (democycline), 100 (DC), 4 (MC), 5 (OTC) and 1 (TC). Serum, urine and egg LODs are 100 for TC [32]. Assay time is 12–15 min [33]. The receptor site of the Charm II binds a functional group of the drug, rather than a side chain, as with immunoassay tests. This allows detection of a class of antibiotics by binding at a single receptor site. Results of the Charm II test have been confirmed using MIT assays for chlortetracycline. Nine samples positive for tetracycline with the Charm II were confirmed positive using a MIT [34]. However, Collins-Thompson *et al.* [35] reported that 40 of 48 milk samples positive for tetracycline by the Charm II test were negative by disc assay, and 8 showed indistinct zones of inhibition. Increased sensitivity of the Charm II and a possible unknown interfering factor were suggested [35]. Charm and Chi [33] reported a 2.3% incidence of false positives for tetracycline in milk and Senyk *et al.* [36] reported no false positives for tetracyline in milk. Charm II has been evaluated as a confirmatory method for positives from microbial screening assays [34,37].

2.9. Thin-layer chromatography–bioautography

Various separation procedures combined with bioautography have been reported and are a blend of physicochemical and bacterial growth inhibition techniques. These include paper chromatography–bioautography [38] TLC-B [23,39–41], and electrophoresis–bioautography [42]. TLC-B is based on selective tissue extraction followed by TLC. The developed TLC plates are placed on a bacterial growth medium seeded with *B. subtilis*. The location of zones of inhibition are used to identify specific antibiotic residues. The sensitivity of the method can be adjusted and antibiotic residue recovery is quantitative. TLC-B provides a multiresidue detection method and can be used to identify individual antibiotics within a class of antibiotics [39]. It has been used in Canada since 1984 for the confirmation of positive in-plant tests [18]. Neidert *et al.* [39] reported minimum detectable amounts in fortified muscle samples (ng/g) as 15 (CTC) and 30 (OTC and TC) as determined by the minimum amount causing visible inhibition zones on 100% of tests at that level. MacNeil *et al.* [23] reported TLC-B lacked the sensitivity of HPLC for OTC but was of equal sensitivity with MIT. STOP had greater or lesser sensitivity than TLCB depending on the growth medium used [23].

2.10. Electrophoresis–bioautography

This procedure can be applied to milk and meat samples and is usually preceeded by a set of MITs. It is a qualitative test but can be made semi-quantitative and is unlikely to allow resolution of related tetracyclines. Antibiotic identification is based on initial MIT results, electrophoretic migration distance, and the appearance of the zone of inhibition [42].

2.11. Enzyme immunoassay

The Cite Probe tetracycline test is a screening test for residues of chlortetracycline, oxytetracycline and tetracycline in milk. It is a competitive immunoassay that visually compares the relative color intensity of a control spot with a sample spot. It is packaged as a self contained kit and can be easily run on-farm. Assay time is 5 min. The limits of detection of tetracyclines in milk are 40 ng/ml for CTC and OTC and 20 ng/ml for TC [43].

The tetracyclines have also been readily detected by other means. In this regard, the fluorescence of tetracyclines under UV light has been used as an indicator of previous tetracycline treatment. It has been used for the detection of OTC residues in bone and injection sites, but fluorescence is non-specific and persists in bone for an extended time after treatment [23,44].

3. METHODS OF ISOLATION

The tetracyclines are congeners of a naphthacenecarboxamide. They have minimal water solubility at pH 7 but will form soluble sodium or hydrochloride salts. The pK_a values of tetracycline itself are 3.3 (hydroxyl group at position 3), 7.5 (dimethylamino group at position 4) and 9.4 (hydroxyl group at position 12). Thus, tetracyclines can exist as zwitterions and are soluble in either dilute acid or

base [6,7]. The bases and hydrochloride salts of these compounds are relatively stable as dry powders. However, most of these compounds rapidly lose antimicrobial activity when in solution, a fact that must be taken into consideration in the design of methodology for their isolation, detection and quantitation. Tetracyclines are also prone to rapid degradation by exposure to light [45–47] and precautions to prevent losses by this route are essential. The tetracyclines are soluble in most alcohols but are quite insoluble in lower solvent strength organics, such as chloroform [6,7]. Their solubility in such solvents is enhanced by conversion to their anhydro- derivatives. However, the compounds doxycycline and minocycline are more lipophilic than their counterparts and are thought to be capable of penetrating mammalian cell membranes, giving them unique pharmacological properties [48].

Isolation of the tetracyclines from aqueous solutions as ion pairs can be conducted by classical counter-current organic solvent extraction of an alkaline medium [6,7]. Isolation from tissues and food products is, however, far more complex. One difficulty in isolating these compounds is associated with the propensity of the tetracyclines to form chelation complexes with metal ions [7,49] and to bind with sample matrix proteins. Indeed, the adsorption and therapeutic effect of the tetracyclines can be diminished by the presence of elevated levels of di- and/or trivalent metals in the diet [48]. Such complexes make the tetracyclines less sensitive to decomposition by light, however [49]. In terms of enhancing the extraction efficiency of a method applied to tissue or other food matrices for the tetracyclines, one should consider the inclusion of a competing chelating agent, such as ethylenediamine tetraacetate (EDTA), citrate or oxalic acid, in order to obtain satisfactory recoveries, especially for low (<100 ng/ml) concentrations. Consideration must also be given to providing the appropriate conditions to minimize protein binding [4]. This may, of course, not be a concern in assays that are performed directly on the sample matrix itself, such as the use of microbial inhibition, immuno- and bacterial receptor assays for the determination of tetracyclines in milk, blood or urine. However, the possible creation of false negative or false positive results from such chelation, protein binding or possible sequestration of the tetracyclines during the performance of such assays has not been thoroughly examined.

3.1. Isolation from milk, urine and blood

The analysis of various tetracyclines occurring in milk, urine and blood, as well as tissues and other foodstuffs, has been accomplished by a number of direct ultraviolet (UV), fluorometric and biological assays [6,7,50,51]. However, these methods lack specificity, although providing a degree of screening capability and simplicity for the research setting, wherein the drug administered is known. Analyses have also been performed by the application of HPLC and TLC after the isolation of the drugs using the more classical countercurrent extraction methods [6,7] as well as a variety of solid phase extraction (SPE) techniques. Several methods have also utilized direct injection of sample supernatant or following protein precipitation [52]. Of further interest are two more recent methodologies that may offer some advantages in comparison to these approaches.

In 1986, Tyczkowska and Aronson [53] reported a multi-tetracycline drug residue isolation method for serum from a variety of animal species utilizing Centricon-30 molecular mass cutoff (30 000) filters. In this process OTC, MC, TC and DC were isolated with recoveries of 76–103% via treatment of the sample (500 μl) with 500 μl mixture (1:1) of LC mobile phase (Na_2HPO_4, phosphoric acid, methanol, acetonitrile, triethylamine and water) and 2% of 85% phosphoric acid in a microseparation system. The sample was then centrifuged for 50 min and assayed by HPLC. A similar approach has been used to isolate DC from bovine and swine serum and urine. Riond *et al.* [54] diluted serum or urine samples with acetonitrile–phosphoric acid–water (20:2:78) and centrifuged the samples through 30 000 (serum) or 10 000 (urine) molecular mass cutoff filters. This method was also applied to the isolation of OTC, TC and CTC from milk by Thomas [55], wherein samples were diluted with an EDTA–phosphate buffer, and filtered through a similar type of molecular mass cutoff (25 000) filter, as described above. After centrifugation for 60 min the milk solids were resuspended and centrifuged for an additional 40 min. Recoveries ranged from 89–97% over the range of concentrations examined (50–1500 ng/ml). Although Tyczkowska and Aron-

son [53] did not utilize chelating agents for serum, they are essential to the isolation process when using milk as a matrix.

A second approach for the isolation of the tetracyclines TC, OTC and CTC from milk was reported by Long *et al.* [56], and utilized a method called matrix solid-phase dispersion (MSPD). In this process the milk sample (500 μl) was blended with a mortar and pestle with 2 g of octadecylsilane (C_{18}, ODS) derivatized silica (40 μm, 60 Å pore size, 18% load, endcapped) and 0.05 g each of EDTA and oxalic acid. The blend was transferred to a column and the column was eluted with 8 ml of hexane and 8 ml of ethyl acetate–acetonitrile (1:1, v/v). The latter eluate was evaporated to dryness, suspended in LC mobile phase (0.01 *M* oxalic acid–acetonitrile, 7:3, v/v), centrifuged and filtered for LC analysis. Recoveries ranged from 64–93% over the range of concentrations examined (100–3200 ng/ml).

3.2. Isolation from tissues

Isolations of tetracyclines from various tissues have followed the classical approach of repeated homogenization of relatively large quantities of sample in the presence of an extracting and/or denaturing solvent. The resulting homogenate supernatant is then often put through a series of sample manipulations to remove co-extracting materials while retaining a high recovery for the target tetracycline(s). For example, Onji *et al.* [57], minced 20 g of sample (bovine muscle or fish) and homogenized with 100 ml of 1 *M* HCl for 5 min. The supernatant obtained after centrifugation was filtered through 5 g of Celite. The pellet was re-extracted with 50 ml of 1 *M* HCl, centrifuged, filtered and the combined filtrates poured onto an Amberlite XAD-2 column. The column was washed with 200 ml of water and then eluted with 100 ml of methanol. The methanol was reduced in volume and the filtered sample was analyzed by HPLC. Recoveries for TC, OTC and CTC were between 67 and 83%.

In a similar approach, Moats [58] homogenized 25 g of tissue (bovine or porcine muscle, liver and kidney) with 75 ml of 1 *M* HCl. An aliquot (8 ml) of the homogenate was mixed with acetonitrile (32 ml), allowed to stand and then decanted with filtration. An aliquot (20 ml) was then extracted with methylene chloride–light petroleum (b.p. 30–60°C) (20 ml each). The resulting water layer was isolated and its volume was adjusted to 4 ml prior to analysis by HPLC. Recoveries ranging from 71–106% for TC, OTC and CTC were obtained over the range of concentrations (1–10 μg/ml) examined.

Similarly, Oka and co-workers [59,60], have homogenized 5-g samples with three separate portions of extracting solvent (total of 50 ml). However, the supernatant obtained after centrifugation was further fractionated using a C_{18} cartridge followed by washing of the cartridge with 20 ml of water. The tetracyclines (TC, OTC, CTC and DC) were eluted with 10 ml of elution solvent and assayed by HPLC. Recoveries were from 68–95% for the various compounds examined at a level of 1 μg/ml in cattle and swine muscle, kidney and liver.

In a similar effort, Rogstad *et al.* [61] developed an SPE procedure for the isolation of oxytetracycline from fish muscle and liver. In this report, 5 g of minced tissue were mixed with 1 g of EDTA and 5 ml of hexane–dichloromethane (1:3). The samples were repeatedly (3X) homogenized in 20 ml of EDTA–phosphate buffer and the combined extracts were centrifuged. After addition of NaCl and a heating and cooling cycle the supernatant obtained after centrifugation was loaded onto a C_8 cartridge. The cartridge was washed with a water–acetone solution (4% acetone) and OTC was eluted with 5% and 10% water in acetone. Recoveries were in the 89–100% range for the tissues examined.

Bjorklund [62] similarly conducted several (3X) homogenization–extraction (citric acid and Na_2HPO_4 buffer) steps on 2 g of fish tissue (trout muscle or liver), sonicated the extracts and then filtered them through paper filters. The resulting filtrate was fractionated on a Bond-Elut C_{18} column by washing with water (30 ml) and eluting the drug with 10 ml of 0.01 *M* oxalic acid in methanol. Recoveries ranged from 77–96% for the isolation of OTC, TC, CTC and DC from salmon liver and muscle.

Reimer and Young [63] have reported a nearly identical SPE method for OTC, TC and CTC in salmon muscle tissue that is based on the reports by Oka and co-workers [59,60]. In this method 5 g of tissue are repeatedly (3X) homogenized with buffer (citric acid and Na_2HPO_4), filtered and loaded onto a Bond-Elute C_{18} column. The column is washed with water and the drugs are eluted using 9 ml of 0.01 *M* oxalic acid in methanol solution. However,

recoveries ranged from 45% (CTC) to 100% (OTC).

Riond *et al.* [54] have applied the use of molecular mass cutoff filters to the isolation of DC from bovine muscle, renal medulla and lung tissues. Minced tissue samples (0.1–0.3 g) were sonicated (30 min) with a solution containing methanol–acetonitrile–phosphoric acid–water (30:10:2:58) and applied to the filtration system, being centrifuged for 30 min. Recoveries ranging from 57% (lung) to 94% (muscle) were obtained for the various tissues.

Long *et al.* [64] have reported a method for the isolation of OTC from fish muscle tissue using MSPD. Muscle tissue, blended as described above for milk, was washed with hexane (8 ml) and the oxytetracycline was eluted with 8 ml of acetonitrile–methanol (1:1). An average recovery of 81% was obtained over the range of concentrations examined (50–3200 ng/g).

4. METHODS OF ANALYSIS

Methods designed to screen for the tetracyclines and to isolate them from foods of animal origin must work in conjunction with procedures designed to separate, detect and confirm the presence or absence of the compound in the sample. This requires that the sensitivity of the screening test and the determinative or confirmatory test be compatible. Consideration must also be given to the possibility that a response from an immunoassay or receptor assay could be the result of the presence of several drugs within a class and that the subsequent instrumental analysis aplied to such a sample would make this fact discernable. Several chromatographic methods have been developed that meet these criteria.

4.1. Thin-layer chromatography

Over the last forty years, numerous paper and thin-layer chromatographic methods for the detection and quantitation of tetracyclines have been reported [4,7]. Some of these reports involve the conversion of the tetracyclines to their corresponding anhydro derivatives [3], a dehydration reaction initiated by heating in 1 *M* HCl. This enhances the UV and fluorescence response of the tetracyclines and makes them more readily extractable from aqueous media at an acid pH. This method has been used by the FSIS for the determination of the nature of the tetracycline present in a sample suspected of being violative [2].

More recently, Oka *et al.* [65] have presented optimized methodologies for the TLC analysis of eight tetracyclines using silica gel high-performance and C_8 reversed-phase plates. Detection in the 0.1–0.03 μg range was accomplished using a diazonium salt solution containing Fast Violet B and scanning of the plates using a densiometer. However, this development was not applied to tissue extracts.

As previously mentioned, several TLC methods have been coupled with bioautography [23,39–41], wherein bacteria seeded media is adsorbed onto developed TLC plates to provide zones of growth inhibition at the R_F values of various antibiotics. Although requiring an overnight incubation, this process can be adapted to provide a generic detection method for antibiotics while being relatively specific, with identification being based on R_F values [39]. This same bioautographic approach has also been applied to sample extracts separated by electrophoresis [42].

4.2. High-performance liquid chromatography

This method of analysis is, perhaps, the one most often employed for the purposes of determining the identification and quantity of the various tetracyclines in the greatest variety of sample matrices. As such, several reviews have been published focusing on LC for the separation and detection of tetracyclines [4–8]. There are also legions of publications involving the chromatography of tetracyclines and their epimers in various pharmaceutic preparations, but these are not considered in detail here [66–69]. Nevertheless, the results of such studies and those relating to the analysis of the tetracyclines isolated from tissue matrices are often affected by the same factors and, thus, have relevance.

One of the factors seen consistently in the analysis of tetracyclines by HPLC is again related to their propensity to form complexes. The tetracyclines as a group are affected by the presence of metals and the presence of free silanols on silica-based solid supports used for HPLC, TLC or for SPE [4–8]. Complications involving free silanol content were more inherent in the derivatized solid support materials sold or prepared for use in studies during the late 1970s to mid-1980s and have been overcome to

a large degree by different manufacturing processes, such as end-capping, the inclusion of citrate, oxalate and/or EDTA in mobile phases, and the availability of all-polymer-based solid supports. Difficulties with metal chelation can be controlled by the use of chelating agents, as given above, or by intentionally complexing the tetracyclines with a metal to reduce competition for such binding. Such chelation can be used to the analyst's advantage since it may also enhance subsequent UV and fluorescence detection and can also provide a molecule that is more electrochemically active and detectable [3,4,7].

Since the tetracyclines have several pK_a values and can exist as zwitterions [6,7] one must consider the control of mobile phase pH in the development of an analytical method. The ability to undergo ion formation also makes the use of ion pairing reagents for the separation and analysis of the tetracyclines practical and several methods have been offered that utilize this approach [4–8]. The maximum and minimum allowable pH values for HPLC instrumentation, effects on column lifetime and that of related components, as well as the nature of the effect of pH on other co-extractants in the sample for analysis, all tend to control the pH that is determined to be optimal in such analyses. With tetracyclines, however, one must also consider the fact that these compounds are susceptible to epimerization over the pH range of 2–6 and that they are generally unstable at acidic and basic pH values [6–7]. Thus, the storage of samples for long periods of time on autosamplers in acidic or basic solutions, especially if unprotected from light, should be avoided.

The tetracyclines have been analyzed via separation on reversed-phase (C_8, C_{18}) derivatized silica solid supports, all-polymer- or resin-based non-ionogenic solid supports as well as ion-exchange solid supports. Of these, the reversed-phase systems, especially C_{18}, have found the most application for the widest range of tetracycline drugs and sample matrices (Table 1). Many of the methods presented are based on those developed by Oka and co-workers [59,60], and utilize a 0.01 *M* oxalic acid–acetonitrile–methanol mobile phase at pH 2.0. This simple isocratic system has advantages over those using gradients or requiring more complex mobile phase compositions in not requiring a re-equilibration time and eliminating the number of reagents in specific combinations required for adequate analysis.

Most HPLC methods for the analysis of tetracyclines have employed fixed, variable-wavelength or diode array UV detectors for sample monitoring. The relatively high levels of these drugs that are allowed to occur in tissues (0.1–4.0 μg/ml in the USA) makes this method of detection adequate for most applications. The extinction coefficients for the tetracyclines are relatively large and the monitoring of samples separated by HPLC at wavelengths ranging from 350–380 nm can give detection into the low tens- of nanograms/g or ml of sample range. The ability to monitor at this wavelength also endows such assays with a degree of specificity, depending on the nature of the matrix and the co-extractants present. This provides the ability to obtain relatively clean chromatograms, which in turn reduces signal-to-noise ratios and allows one to have relatively short analysis times (8–16 min/sample) without the need to conduct time and solvent consuming gradient analyses.

Although the tetracyclines possess the ability to be detected by fluoresence, few methods employing this technique have been developed. However, Blanchflower *et al.* [70] have presented a technique for the HPLC–fluorescence detection analysis of chlortetracycline in tissue samples. This approach is based on the conversion of the CTC to its more highly fluorescent iso derivative. An excitation wavelength of 340 nm and an emission wavelength of 420 nm were utilized. However, the conversion process required some 2.5 h after extraction of the sample and limits of detection were not significantly improved over those seen with UV.

The tetracyclines are also electrochemically active [7,71] but little in the way of application of this method of detection to these drugs as residues in food animal tissue or matrices has been published.

4.3. Gas chromatography

There are a limited number of publications involving the use of gas chromatography for the analysis of tetracyclines [72,73]. Tsuji and Robertson [73] were the first to apply trimethylsilylation to the tetracycline molecule, obtaining sufficiently volatile and stable derivatives for packed-column gas chromatography–flame ionization analysis. The method was also shown to be capable of performing quanti-

TABLE 1

REPRESENTATIVE METHODOLOGY FOR HPLC ANALYSIS OF TETRACYCLINES FROM VARIOUS SAMPLE MATRICES

grad. = Gradient analysis; ACN = acetonitrile; DMF = dimethylformamide; MeOH = methanol; THF = tetrahydrofuran.

Drug(s)	Matrix	Column type	Mobile phase	Detection	Analysis time (min)	Ref.
TC, OTC, CTC	Bovine tissues, Bovine, swine serum	PLRP-S, 5 μm	grad.; H_3PO_4–MeOH–ACN (30:50:20)	UV, 355 nm	22	58
OTC, MC, TC, DC	Serum	Phenyl Spheri-5 MPLC	ACN–triethylamine–Na_2HPO_4–H_3PO_4–MeOH (1.5:0.5:8.6:2:10)	UV, 267 nm	23	53
OTC	Fish	ODS Hypersil	$(NH_4)_2HPO_4$–diethanolamine–ACN–DMF–H_3PO_4 (8.1:0.05:1.9:0.6, pH to 2.5 using H_3PO_4)	UV, 365 nm	8	79
OTC, TC	Fish	Hypersil SAS, 5 μm	Citrate–Na_2EDTA–ACN (34.5:0.5:15)	UV, 370 nm	8	80
OTC	Fish	Supelcosil LC-18DB, 5 μm	Phosphate buffer (pH 2)–ACN–THF (81:10:9)	UV, 357 nm	10	61
OTC, TC, CTC, DC	Fish	ODS-Spheri-5	ACN–DMF–0.01 *M* oxalic acid (22:6:72)	UV, 355 nm	8	62
TC, OTC, CTC	Milk	NovaPak C_{18}	grad.; 0.01 *M* oxalate–MeOH–ACN (70:8:22)	UV, 360 nm	16	55
DC	Bovine, porcine tissues, body fluids	Phenyl Spheri-5	ACN–triethylamine–0.08 *M* Na_2HPO_4–H_3PO_4–MeOH (2.5:0.5:73.8:1.7:22.5)	Diode array, 235–380 nm	12	54
TC, OTC, CTC	Milk	MicroPak C_{18}, 10 μm	0.01 *M* Oxalate–ACN (70:30)	Diode array, 365 nm	8	56
MC, OTC, TC, CTC, Methacycline, Dimethyl-CTC	Bovine plasma, kidney	μBondapak C_{18}	Na_2HPO_4–ACN–DMF–ethanolamine (76:24:6:0.5)	UV, 254 nm	12	81
TC-OTC-CTC	Salmon	LiChroCART RP-18	0.01 *M* Oxalate–ACN–MeOH (73:17:10)	Diode array, 355 nm	15	63
CTC, OTC	Swine tissues	LiChrosorb RP-8	0.01 *M* Oxalate–ACN–MeOH (1:1.5:2.5)	UV, 350 nm	10	77
OTC, CTC, DC	Bovine, porcine tissues	LiChrosorb RP-18	0.01 *M* Oxalate–ACN–MeOH (1:1.5:2.5)	UV, 350 nm	12	59,60
OTC	Catfish muscle	MicroPak C_{18}, 10 μm	0.01 *M* Oxalate–ACN–MeOH (70:27.5:2.5)	Diode array, 365 nm	6	64

tative analyses, having good agreement with microbial inhibition assays. However, the formation of the derivatives was sensitive to a number of variables.

5. MASS SPECTROMETRY: CONFIRMATION OF TETRACYCLINES

There have been few articles published on the application of various mass spectrometric (MS) techniques to the detection, identification and confirmation of the tetracyclines. The various extraction and HPLC methods available make analysis of the tetracyclines by LC–MS a practical concept, but only two articles applying this approach to the confirmation of DC [74] and to OTC, TC and CTC [75] have been found by the authors. Nevertheless, such methods are presently being applied by governmental agencies for the confirmation of tetracycline positives that are found to be in violation of regulatory limits (personal observation).

One notable exception is the MS–MS method reported by Traldi *et al.* [76], for the analysis of OTC in milk and muscle tissues using a single-ion monitoring (SIM), collisionally activated decomposition (CAD), mass-analyzed ion kinetic energy spectrometric (MIKES) approach. In this method, crude extracts from fortified (1–10 ng/g or ml) milk or muscle, obtained by ethanol extraction and, centrifugation, evaporation and resolubilization in ethanol, were directly introduced into the instrument and analyzed by the SIM-CAD-MIKES-MS–MS method. Limits of detection were in the low ng/g range and adequate data were obtained to permit confirmation of identity of the species examined.

6. METHOD DEVELOPMENT AND EVALUATION CRITERIA

The choice of methods for a given application is driven by a variety of factors. For sample extraction these factors include the following;

(a) The sample size necessary to obtain a given limit of detection or determination for the analytical instrumentation available.

(b) The nature of the matrix and the availability of existing methods to deal with the isolation of the target molecules from the specific matrix.

(c) The necessary specificity of the isolation technique; *i.e.,* the ability to isolate a single compound within a drug class, several compounds within a drug class or to isolate several drug classes from a single sample.

(d) Sample numbers and turn-around time.

(e) The cost of the method, including supplies and disposables, time and overall labor involved and the costs of the instrumentation that will be applied.

All of these factors are interrelated and are dependent on what the analyst must or needs to accomplish. However, the simplest method of extraction is the one that requires no sample manipulation. These are the methods that extract the drug directly from the sample matrix by means of specific or selective antibodies or receptors. These assays, as described above, can be configured to simultaneously isolate, quantify and, in some cases, provide a preliminary identification of a variety of different compounds. Such tests are most applicable to aqueous solutions of the drugs, such as milk, urine, blood or blood fractions and to tissue homogenates or solubilized extracts from tissues or other food products. Despite their power, such approaches are, however, also prone to interference from naturally occurring compounds in the sample matrix and to the possible concentration of such interferences from extracts. Interferences that also bind to antibody or receptors can lead to false positive results. Such interferences may be naturally occurring compounds or other drugs or their metabolites that occur in the sample but are not recognized as having cross-reactivity in the assay. A further concern is that the drug may remain bound to sample proteins or be in complexes that do not bind with a given antibody for their detection, leading to false negative results or a reduced response from what is actually a higher than detected level. This is especially of concern for drugs like the tetracyclines that possess both high protein binding and complex formation potential.

For these reasons, and for purposes of performing more exacting quantitation and identification of the detected residues, one must often perform sample manipulations subject to the considerations listed above, even for immuno- or receptor assays. For the tetracyclines the following approaches have been delineated;

(a) Protein precipitation–direct HPLC injection;

mainly for aqueous samples, such as serum, but has also been applied to milk and urine. Organic solvents or acids used for precipitation must be removed prior to use of the supernatant in an immunoassay.

(b) Counter-current and/or homogenization–solvent extraction; classical solvent–matrix distribution conducted by extraction of an aqueous sample and/or by the homogenization of the sample in the presence of an extracting solvent. Emulsion formation can be a problem and centrifugation and repeated re-extraction of the pellet obtained from the samples is often required. Again, organic solvents and strong acids would interfere with the use of the isolate in an immunoassay format.

(c) Solid-phase extraction; except for aqueous sample matrices, requires pre-solubilization or extraction, as described above, prior to addition and fractionation on the column. Drugs isolated using organic solvents could be assayed by antibody methods after evaporation of the solvent and resolubilization in an appropriate assay buffer or reagent.

(d) Ultrafiltration; as above, also requires pre-solubilized sample extract if other than an aqueous sample. However, aqueous filtrates could be used directly in immuno or receptor assays.

(e) Matrix solid-phase dispersion; can be applied to aqueous or solid samples. Organic solvent used for elution would have to be removed before extract could be used in an antibody or receptor based assay.

All of these methods may prove useful for a given application. However, a further consideration that has obtained greater significance is the recognition that methods that generate large quantities of solvent or other wastes are becoming too expensive and too hazardous to the environment to perform. Thus, extraction methods that use large sample sizes and, thus, require large volumes of organic solvent to adequately perform residue isolation are becoming increasingly unacceptable. For this reason alone many of the classical homogenization–extraction methods will have to be eliminated and procedures such as direct injection, ultrafiltration, SPE and MSPD will have to be implemented.

Any method for the isolation of drugs must take these factors into consideration. For the tetracyclines one must also develop the analytical methodology to eliminate pH and light-induced decompositions or rearrangements as well as assure that complications from protein binding and chelation with metals is eliminated or controlled. The exclusion of light, the use of appropriate buffers to control pH and the ionization state of the drug and the inclusion of chelating agents such as citrate, EDTA and oxalate should provide appropriate control for these factors and provide adequate recovery and ability to analyze tetracycline containing samples.

These factors are not only the case in the extraction methodology but also in the methods applied to the quantitation and identification of the tetracyclines. As described, several of the immunoassays available in the market are quite specific and can be quantitative. The use of such tests for such purposes thus eliminates much of the sample manipulation and difficulties that arise as described above. Similarly, where the particular tetracycline to be quantitated is known, as in various tetracycline research scenarios, one may find simple UV or fluorescence monitoring of the sample to be adequate. Nevertheless, there will be the need at some juncture to provide a less equivocable analysis of the residue. For the tetracyclines the method of choice would be HPLC with UV detection, using a variable-wavelength or diode array detection system. Monitoring by UV at wavelengths between 350 and 380 nm provides adequate detection and a degree of selectivity over co-extractants that allows for excellent separation and short run times (8–16 min) for one or a number of tetracyclines in a single analysis and in a variety of matrices.

Although many HPLC techniques have been described over the years, one must exclude from consideration any strict adherence to LC methods reported prior to 1985. This fact is in large measure due to the very different nature of the solid supports available today when compared to those of the past. More recent methodology [Table I] has shown that a reversed-phase C_{18} column and an isocratic mobile phase at an acid pH (*ca.* pH 2), containing oxalic acid, acetonitrile and methanol can provide more than adequate methodology for the separation, quantitation and identification of the various tetracyclines and has been used to perform large surveys for tetracycline contamination in the food supply [77]. Such methodology can complement microbial or immunoassay tests in that they have

short analysis times overall and LODs in the same concentration range.

Although little is available in the literature regarding the confirmation of tetracyclines isolated from food matrices, it is a practical matter that gas chromatography [72,73] and LC–MS [74,75] are applicable to such endeavors. Gas chromatographic analysis can be accomplished by trimethylsilylation of the tetracycline molecule and, in combination with MS, especially in various selected ion monitoring modes, should provide more than adequate sensitivity and selectivity for performing confirmations for suspect samples. The CAD-MIKES-MS–MS method offered by Traldi *et al.* [76], provides a highly sensitive and specific technique for tetracycline analysis. However, this can be little more than an experimental MS curiosity since the availability of the instrumentation to perform such analyses is out of the reach of most laboratories.

7. EVOLVING ANALYTICAL METHODS

There is little doubt that the direction being taken for the determination of drug residue contamination and possible violations in foods of animal origin is that of the increased utilization of immuno and receptor based assays. These assays are finding increasing application to the direct screening of milk, blood and tissue transudate or homogenate for the more rapid identification of suspect or violative samples. This technology will be extended to include the development of and to make more readily available the use of immunoaffinity chromatographic columns or discs [78]. However, one cannot obtain adequate data to identify and, in some cases, quantify residues that produce responses in such assays alone. There will remain a need to provide techniques for the chromatographic fractionation and identification of the various residues. Where sample homogenization or disruption, coupled with a degree of fractionation, is needed methods based on MSPD, SPE and ultrafiltration can be applied. These methods, like the immunoassys, are also amenable to automation and are, thus, compatible in terms of sample through-put and turn-around time with the more rapid screening techniques. Advances in LC technology, in terms of in-line extraction, microbore columns and more sensitive and selective detectors, will also prove applicable.

8. CONCLUSIONS

While numerous methods for the isolation, separation, detection and quantification of the tetracyclines occurring as residues in foods of animal origin have been developed, they are rapidly being replaced by antibody-antigen based systems that perform simultaneous extraction, isolation, detection and, in some cases, quantitation. Such approaches are also replacing slower and less specific microbial inhibition assays that have been the backbone of many residue monitoring programs. The new assays must be supported by extraction methods that are capable of performing rapid and reasonably specific isolation techniques, providing rapid identification and/or a degree of confirmation of the residue in question. Depending on the sample matrix involved, there are analytical approaches available to perform such analyses for several possible tetracycline residues in a single sample simultaneously. Further, simple isocratic HPLC analysis methods for all or most of these compounds simultaneously are also available and can be conducted in a relatively short analytical time frame per sample. Thus, the combination of evolving immunoassay detection and screening technologies coupled with multi-residue extraction or sample preparation techniques and rapid HPLC analysis should provide the necessary speed and accuracy for the monitoring, regulation and control of tetracycline residues in foods of animal origin.

9. ABBREVIATIONS

ACN	Acetonitrile
AOAC	Association of Official Analytical Chemists
CAD	Collisionally activated decomposition
CAST	Calf antibiotic and sulfa test
CTC	Chlortetracycline
DC	Doxycycline
DMF	Dimethylformamide
EDTA	Ethylenediamine tetraacetate
FAST	Fast antibiotic screen test
FSIS	Food Safety and Inspection Service
grad.	Gradient analysis
HPLC	High-performance liquid chromatography
LAST	Live animal Swab test

LC	Liquid chromatography
LOD	Limit of detection
MC	Minocycline
MeOH	Methanol
MIKES	Mass-analyzed ion kinetic energy spectrometry
MIT	Microbial inhibition test
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
OTC	Oxytetracycline
SIM	Single-ion monitoring
SPE	Solid-phase extraction
STOP	Swab test on premises
TC	Tetracycline
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TLC-B	Thin-layer chromatography–bioautography
USDA	United States Department of Agriculture
UV	Ultraviolet

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Review

Chromatographic analysis of Maillard reaction products

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ABSTRACT

This paper reviews the products of the Maillard reaction (non-enzymic browning) which are most frequently analysed in food quality control. In particular, high-performance liquid chromatographic (HPLC) methods are reported for 5-(hydroxymethyl)-2-furfural and ε-N-2-furanylmethyl-L-lysine, early indicators of the Maillard reaction, ε-pyrrolelysine, an advanced indicator of the Maillard reaction, and free and oxidized N-acetylmethionine, a food additive used to overcome the degradation of amino acids. Some analytical methods are evaluated in comparison with HPLC.

CONTENTS

1. INTRODUCTION

There are several types of non-enzymic browning. One of them is caused by Maillard reaction [1–12], which is initiated by the combination of an amino acid with a sugar according to the following general route: sugar + amino acid → Amadori compounds → pigments [13–24]. The amino acid may be present in the food either free or as part of a protein. The sugar must contain a reactive carbonyl group.

The pigments formed are flavourful and brown or black in colour. Contrary to popular opinion, the Maillard reaction does not require high temperatures; thus sugars and amino acids, even when stored at refrigeration temperatures, can show signs of non-enzymic browning. The reaction rate, however, increases markedly with increase in temperature and with decrease in moisture level, although browning can also proceed in aqueous solution [15–30].

A second type of browning is caramelization.

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When sugars are heated at high temperatures they turn first brown and then black. This reaction involves the dehydration or removal of water from the sugar, which by various reactions results in the formation of furfural. A third type of browning is caused by ascorbic acid, which, once oxidized, forms the same type of compounds as already described.

All this can only give a very general idea of one of the most complex and most investigated phenomena of food chemistry. High-performance liquid chromatographic (HPLC) methods are reported here for determining some Maillard reaction products which are of paramount importance for the evaluation of parameters related to food processing and storage (time and temperature abuses) [31–34].

2. EARLY INDICATORS OF THE MAILLARD REACTION

2.1. 5-(Hydroxymethyl)-2-furfural (HMF)

HMF is an intermediate formed in the early stage of non-enzymic browning. In particular, its amount in canned products has been suggested as giving an indication of the extent of damage to the product caused by excessive heat during processing or subsequent storage. Thus the HMF content may be used as a quality criterion for a wide range of products (fruit juice, tomato products, soft drinks, honey etc.) [35–39]. Two chromatographic procedures are available for HMF determination.

The sample for analysis is diluted according to its concentration [1:4 (v/v) for a fruit juice], 2 ml each of Carrez I and II reagents are then added and the volume is made up with distilled water. The Carrez clarifying agent consists of two aqueous solutions, one of 15% (w/v) potassium hexacyanoferrate (II) (Carrez I) and the other of 30% (w/v) zinc sulphate (Carrez II). The sample is centrifuged for 2 h at 40 000 *g* and 1 ml of clear supernatant is pipetted into a syringe and filtered through a 0.45-μm disc filter before injection.

The chromatographic conditions for the first method [40] can be specified as follows: column type, C_{18} (250 mm × 4 mm I.D.) with a mean particle diameter of 10 μm; eluent, water–methanol (90:10, v/v) which has been previously degassed and filtered through a 0.45-μm filter, with isocratic elution at 1.5 ml/min; detection, UV at 285 nm and injection volume, 10 μl. The limit of detection of the method is 0.1 mg/l. A typical result is presented in Fig. 1.

The second method [41] was developed to determine both furfural (another intermediate particularly important with low-pH foods) and HMF. In this version the chromatographic conditions can be specified as follows: column type, C_{18} (100 × 8 mm I.D.); eluent, acetonitrile–water (15:85, v/v) (degassed and filtered) with isocratic elution at 2.0 ml/min; detection, UV at 280 mm and injection volume, 20 μl. The results are exemplified in Fig. 2.

2.2. ε-N-2-furanylmethyl-L-lysine (FUR) (furosine)

Furosine forms during the acid hydrolysis of ε-deoxyfructosyllysine (ε-DFL), the most stable Amadori compound in early Maillard reaction. The level of FUR in heat-treated products is related to processing and storage conditions and may be further converted into nutritionally unavailable lysine [42–53]. Hence FUR may represent a suitable indicator of the quality of various products, in particular dairy products, dry pet foods, snack bar and dry gravies.

The sample for analysis is treated in the following

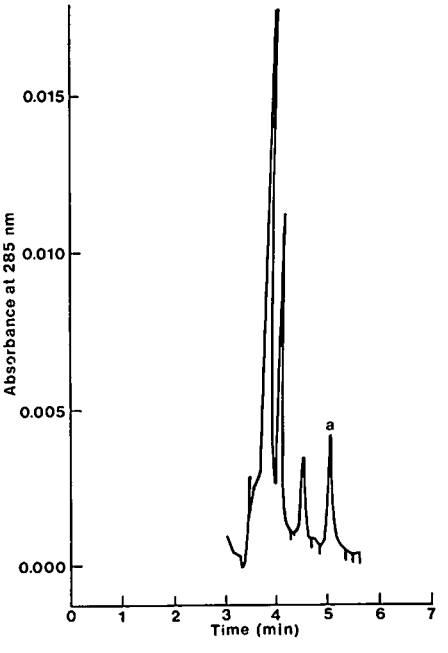

Fig. 1. HPLC profile of tomato paste on a C_{18} silica column. Peak a = HMF (5.1 min).

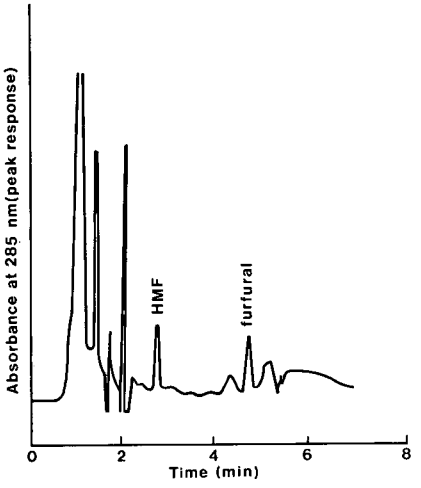

Fig. 2. HPLC profile on a C_{18} silica column of orange juice stored for 24 weeks at 40°C.

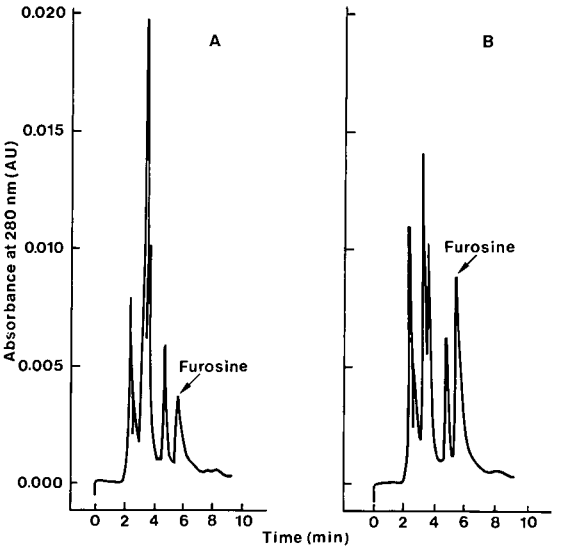

Fig. 3. HPLC profiles of acid hydrolysates of powdered meal replacer products (chocolate flavour) on a C_8 silica column. (A) Control; (B) 20 weeks of storage at 45°C.

way: 0.1–0.5 g of sample containing *ca.* 20 ml of protein is weighed into a 25-ml screw-capped vial, 10 ml of 6 *M* HCl are added and the mixture is heated in an air oven at 110°C for 20 h. Before injection, the filtered hydrolysate is diluted with 3 *M* HCl to a protein concentration of 1–2 μg/μl. The chromatographic conditions can be specified as follows [43]: column type, C_8 (250 × 4.6 mm I.D., 10 μm); eluent, 0.5% acetate buffer prepared by dissolving sodium acetate in water purified in a Milli-Q system (Millipore, Bedford, MA, USA) and adjusting the pH of the solution to 4.3 with glacial acetic acid; elution is carried out isocratically at 2 ml/min; injection volume, 50 μl and detection, UV at 280 nm. The limit of detection of the procedure is: 0.1 μmol/g. A typical chromatographic separation is shown in Fig. 3.

3. DETERMINATION OF ε-PYRROLELYSINE IN PROCESSED FOOD AS AN INDICATOR FOR THE ADVANCED MAILLARD REACTION

During the first stage of the Maillard reaction, Amadori compounds (1-amino-1-deoxy-2-ketoses) form. The bound amino acid in these compounds is not available as a source of amino acid, which results in a decrease in the nutritional value of foods [54–57]. To date Amadori compounds have been investigated in relatively few biological products owing to the tedious procedures required for their isolation and purification. Hurrel and Carpenter [58] called the "early Maillard reaction" products those leading to the formation of Amadori compounds and "advanced Maillard reaction" products those formed in the subsequent steps. ε-Pyrrolelysine, a glucose lysine Amadori compound, has been identified in the water-soluble fraction of many processed foods. Furosine forms during early Maillard reactions and decomposes as the reactions progress. Furosine determination alone cannot be used to evaluate the extent of Maillard reaction especially in long-term stored products. The following method was applied to various stored samples such as non-dairy cream, dry dog food, instant gravy, powdered meal replacer, dried milk, hot cocoa mix and semi-moist sauce.

The measured ε-pyrrolelysine level in each product reflected the shelf-life and storage conditions closely. Moreover, the ε-pyrrolelysine level maintained its upward trend even under severe heat treatment (110°C), which suggests that it may serve as an indicator of advanced Maillard reaction. ε-Pyrrolelysine standard can be synthesized according to Nakayama *et al.* [56].

The sample preparation method is different for

different products and two examples are given here, for powdered foods and non-dairy cream.

For powdered foods 1 g of sample is weighed in a 20-ml screw-capped vial and after addition of 8 ml of warm (60–65°C) water is vortex-mixed for 1 min. The solution is then deproteinized by the addition of 1 ml of $ZnSO_4$ solution (33.4 g per 100 ml) and 1 ml of potassium hexacyanoferrate (II) solution (17.29 g per 100 ml) and centrifuged at 28 000 *g* for 15 min. The supernatant liquid is filtered through a Millipore filter (0.45 μm) and analysed by HPLC. Results are shown in Fig. 4.

With non-dairy cream samples, a small glass-wool-plugged funnel is placed on top of a 20-ml screw-capped vial, 1 g of the sample is weighed and placed in the funnel and 3 × 4 ml of $CHCl_3$ are added. With each addition, the $CHCl_3$ is allowed to drain slowly into the vial and then discarded. The sample is air-dried to remove the remaining $CHCl_3$. After addition to the sample of warm (60–65°C) water, a disposable pipette is used to push the glass-wool at the top of the funnel down into the vial and the same pipette is used to remove the remainder of the sample from the funnel. The sample is then deproteinized as described, the funnel is removed and the vial is capped. After vortex mixing, the solution is centrifuged and the supernatant is filtered through a Millipore filter (0.45 μm) and analysed by HPLC. Results are shown in Fig. 5.

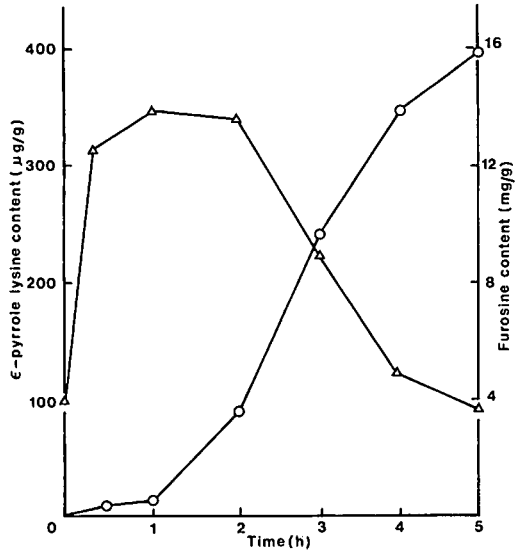

Fig. 4. (○) ε-Pyrrolelysine and (△) furosine contents of powdered meal replacer (vanilla flavour) heated at 110°C for up to 5 h.

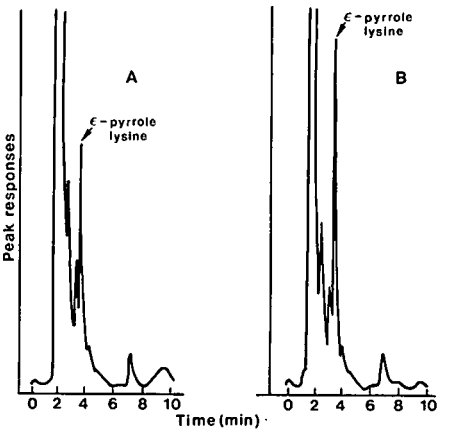

Fig. 5. HPLC of non-dairy creamers on a C_8 silica column. (A) 8 weeks of storage at 22.2°C; (B) 8 weeks of storage at 45°C.

The separation of ε-pyrrolelysine is carried out under the following conditions: column type, C_8 (250 × 4 mm I.D., 7 μm); eluent, 0.5% acetate buffer prepared by dissolving sodium acetate in Millipore-filtered water and adjusting the pH of the solution to 4.3 with glacial acetic acid; elution is carried out isocratically at 1.6 ml/min; detection, electrochemical with the guard cell voltage set at 0.7 V; applied voltage, 0.55 V.

4. SEPARATION OF BROWNING PIGMENTS

The brown pigments isolated from the model reaction between aldoses and amino acids [54,59–62] proved to be insoluble in common organic solvents; some were readily soluble in water, some slightly soluble and others insoluble. The soluble pigments were isolated by dialysis; the proportion of non-dialysable to dialysable pigment increased as the reaction proceeded. Pigments can also be isolated by passing the reaction mixture through a column of the dextran molecular sieve Sephadex G-25 Pharmacia (Uppsala, Sweden). The best separations achieved were similar to those obtained by dialysis.

As far as natural systems are concerned, a brown pigment was isolated from darkened diced apricots [63] by extracting the fruit with 50% hot acetone and after extraction with 95% and 75% ethanol. The pigment was precipitated with acid, or by addi-

TABLE 1

TERNARY SOLVENT SYSTEM FOR THE SEPARATION OF THE BROWNING PIGMENTS FORMED IN STORED FRUIT JUICE

Time	Water (%)	Acetonitrile (%)	Tetrahydrofuran (%)
0	99	0	1
5	97	0	3
38	89.5	3.5	7
78	70	5	25
98	55	5	40
103	0	90	10
108	0	90	10
111	99	0	1

tion of acetone, and was purified by dissolution in dilute alkali and precipitation with dilute acid. The yield was 5–7% of the dry mass of the fruit. The type of amounts of pigments are container dependent [64,65].

Before analysis the sample is treated in the following way: 50 ml of the sample are freeze-dried, the residue is extracted with 30 ml of methanol, filtered through Whatman (Maidstone, UK) No. 42 filter-paper and then made up to volume with methanol in a 50-ml volumetric flask. Samples are filtered through a 0.45-μm filter and stored at -10°C until analysis. The following conditions of the chromatographic separation are applied: column type, C_{18} (150 × 4 mm I.D., 3 μm) and C_{18} precolumn (50 × 4 mm I.D.); eluent, a ternary solvent system consisting of water, acetonitrile and 50% aqueous THF as specified in Table 1; flow-rate, 1.0 ml/min; all gradient rates are accomplished linearly; sample injection volume, 15 μl; detection, UV–VIS at 436 nm. The results of the chromatographic separation are presented in Figs. 6 and 7.

Another category of naturally occurring pigments are the melanoidins from browning prune pulp [66]. Their chromatographic separation can be effected in the following way: a 4.5-g sample of pulp is cut into small pieces and ground for 3 min in an Omni mixer in 60 ml of 50% ethanol. The suspension is left to stand at room temperature for 24 h with occasional shaking and is finally centrifuged at 27 500 *g* for 20 min at 5°C. The extracts are partially purified as follows: 1 ml of 10% lead acetate and 9 ml of 2% acetic acid are added to 10 ml of prune extract and mixed thoroughly. The precipitate formed is removed by centrifugation at 2500 *g* for 5 min, an equal volume of 95% ethanol is added to the supernatant and the precipitate is removed again by centrifugation. The supernatant is concentrated to about 30 ml under vacuum at 37°C to remove the acid and diluted to 60 ml with distilled water. Successive concentration and dilution are repeated until the final pH reaches *ca.* 5.0 to eliminate

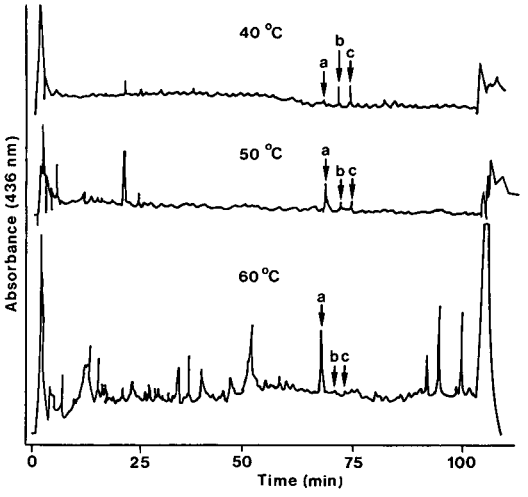

Fig. 6. HPLC on a C_{18} silica column of grapefruit juice browning pigments formed in glass bottles when stored at 40, 50 and 60°C for 12 weeks. a–c = Different browning pigment entities.

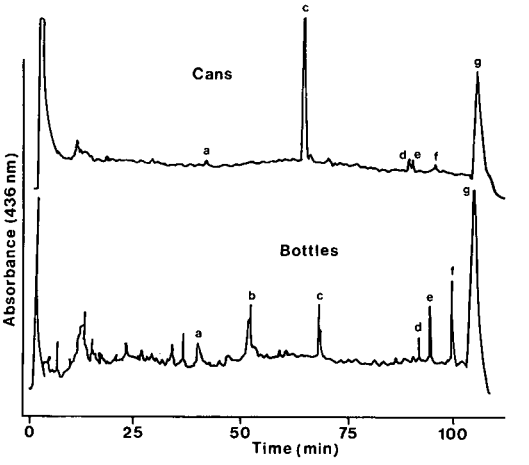

Fig. 7. HPLC on a C_{18} silica column of browning pigments formed in grapefruit juice stored in glass and cans at 60°C for 9 weeks. a–g = Different browning pigment entities.

most of the acetic acid. The pH of the extract is then adjusted to 3.0 with 2 *M* HCl in order to allow the adsorption of the phenolic compounds on Sephadex LH-20. The extracts are then passed through a Sephadex LH-20 column (40 × 2.5 cm I.D.) at a flow-rate of 1 to 2 ml/min. The non-adsorbed compounds are concentrated to 60 ml under vacuum at 37°C. The pigments adsorbed on the column are eluted with about 300 ml of acetone–water (1:1, v/v) and concentrated to 60 ml. The solution containing non-Sephadex-adsorbed compounds are passed through a Dowex 50 (H^+) ion-exchange column (15 × 2 cm I.D.) (Dow Chemical, Midland, MI, USA). After elution with distilled water, the eluate is concentrated to 60 ml. The pigments retained on the Dowex 50 (H^+) column are eluted with 300–400 ml of 1 *M* ammonia solution and the resulting eluate is concentrated to 60 ml.

5. DETERMINATION OF N-ACETYLMETHIONINE (NAM) [67–71]

The protein value of foods limited in methionine content can be improved through fortification with free methionine, but to relieve this deficiency the addition frequently makes the foods impalatable for human consumption. These flavour effects [72,73] are caused by Maillard browning and Strecker degradation reactions, which yield volatile sulphides [74]. Methional (broth-like odour) has been identified as a major product from the Strecker degradation of methionine. The nutritional losses and the flavour problems associated with methionine fortification could be overcome by using a methionine derivative, N-acetyl-L-methionine (NAM), which has been recommended [75] and approved as a food additive [76]. The protected amino group of NAM is relatively stable to Strecker degradation and offers higher sensory thresholds than methionine in amino acid-fortified foods.

The following conditions were developed to determine NAM in model systems: column type, C_8 (25 × 4.6 mm I.D., 10 μm); eluent, phosphate buffer (0.01 *M*, pH 7.0)–acetonitrile (70:30, v/v) with isocratic elution at 2.0 ml/min (retention time *ca.* 4 min); detection, UV–VIS at 254 nm; injection volume, 10 μl. The results shown in Fig. 8 indicate that NAM offers much greater stability than methionine to Maillard browning.

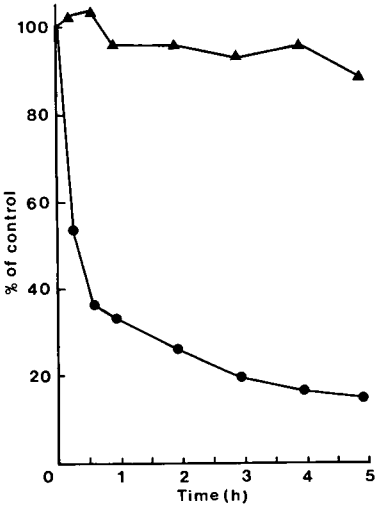

Fig. 8. Recovery of (▲) N-acetylmethionine (NAM) and (●) methionine in a model system containing glucose heated at 95°C.

6. COMPARISON BETWEEN OPTICAL AND CHROMATOGRAPHIC METHODS IN THE DETERMINATION OF SOME INTERMEDIATES OF THE MAILLARD REACTION

6.1. Hydroxymethylfurfural [40]

In the determination of HMF, the commonly employed spectrophotometric method is that of Winkler [77], which is based on the use of particularly toxic reagents such as barbituric acid and *p*-toluidine solutions, to be prepared daily. With this method, the recoveries are highly variable and in any case do not exceed 83%. The first important limitation to the Winkler method is the difficulty in following the colour reaction accurately over time because maximum colour intensity is obtained within an interval of 1–4 min, so its optimum recording is not always possible when large amounts of samples are to be analysed.

The linear regression equation ppm HMF (Winkler) = −3.30 + 0.96 ppm HMF (HPLC) (R = 0.99) can be used to convert data correctly from one method to the other.

Typical results are shown in Figs. 9 and 10.

6.2. Absorbance measurement at 420 nm

Generally, the intensity of browning based on absorbance measurement at 420 nm is largely in agreement with the visual observation of the products

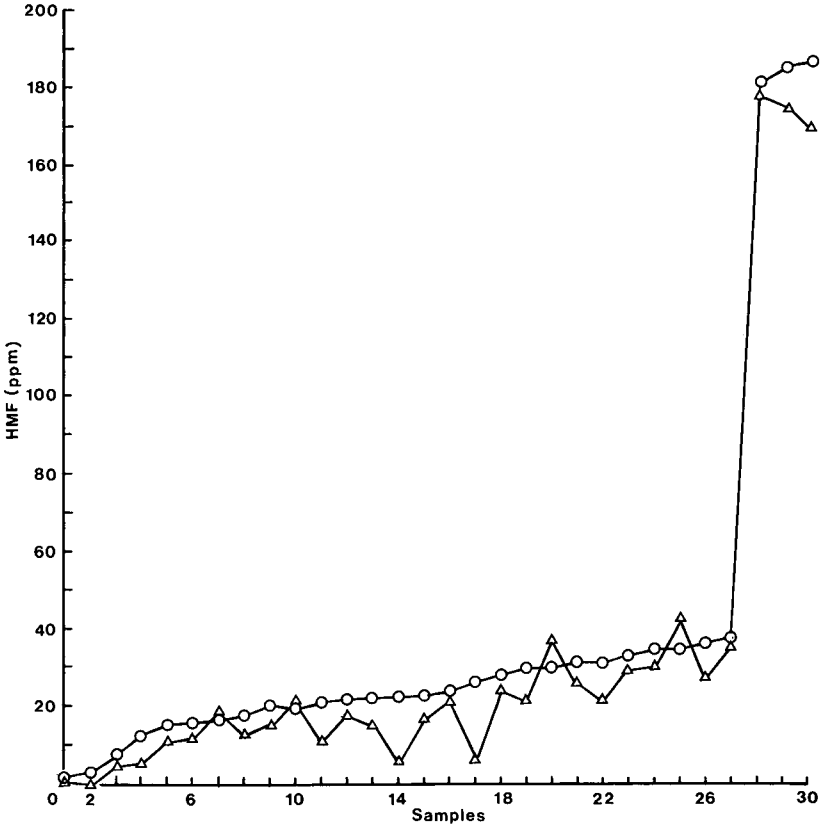

Fig. 9. HMF contents (mg kg^{-1}) obtained by two methods (△ = Winkler spectrophotometric method and ○ = HPLC on a C_{18} silica column) in tomato paste samples of different concentrations.

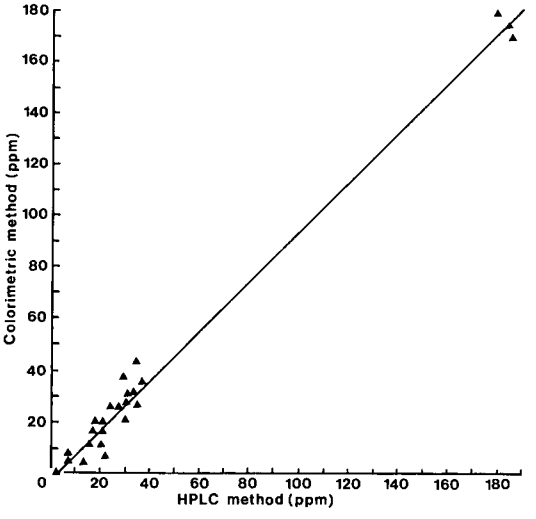

Fig. 10. Correlation between the spectrophotometric (Winkler) and HPLC (on a C_{18} silica column) methods.

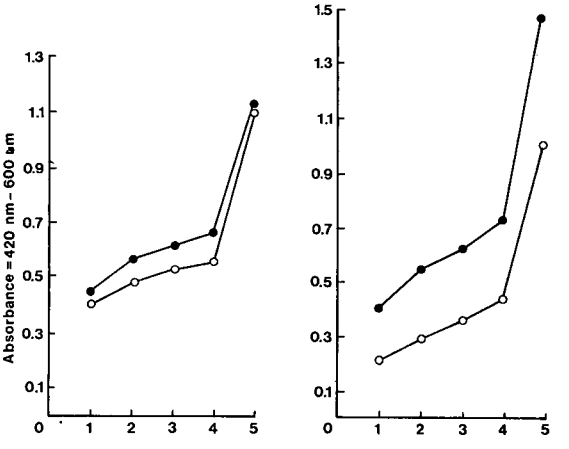

Fig. 11. Correlation between the visual colour tone index (sensorial) and the absorbance of dried apricots. Colour (abscissa): 1 = yellow-golden; 2 = yellow-amber; 3 = light brown; 4 = brown; 5 = dark brown.

(fruit in particular). For fruit juice, extraction is simply carried out by diluting the samples to 2.5°Brix and filtering, whereas with solids, *e.g.*, dried apricots, extraction is carried out on cut samples using 50% ethanol and ultracentrifuging at 30 000 *g* for 20 min at 5°C. Sometimes the absorbance at 420 nm is corrected for turbidity by subtracting the absorbance at 600 nm.

Fig. 11 shows the correlations between visual colour tone (sensorial) index and absorbance (420 and 600 nm).

7. CONCLUSIONS

Further investigations on non-enzymic browning (Maillard reaction) are still required above all to elucidate the numerous and complex reaction mechanisms and the nature and composition of the compounds formed. Studies in progress mainly concern the determination of volatile compounds from interactions in the Maillard reaction and these results will provide a better understanding of some of the chemistry taking place during the cooking of food. Another step to be taken should be towards the development of simple multi-component techniques so as to give a greater number of food manufacturers the possibility of controlling the Maillard reaction by using up-to-date methods. However, there are some chemical parameters that are well suited to the requirements of quality control laboratories as regards the identification of damage caused to food products by the Maillard reaction, and this paper has dealt with the determination of some of these parameters.

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Review

Chromatographic analysis of anthocyanins

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ABSTRACT

Anthocyanins are the red, blue and purple pigments responsible for the coloration of many plants. These pigments have been the subject of many studies due to their importance as a quality indicator in foods and as an important chemotaxonomic indicator for plants. Early work with anthocyanins employed paper chromatographic methods. More recently, high-performance liquid chromatography has been widely applied to the study of these pigments. The objective of this paper is to review the chromatographic methods that have been employed in the analysis of anthocyanins with emphasis on the more recent developments in high-performance liquid chromatographic analysis of anthocyanins as applied to food quality measurement.

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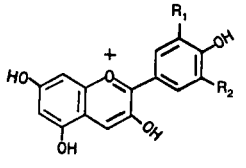
1. INTRODUCTION

The anthocyanins are responsible for the pleasing red, blue and purple colors of most commonly grown fruit varieties and also in some citrus and tropical fruits. The different colors of the anthocyanins are obtained through extents of hydroxylation, methylation, and glycosylation. Chemically, the anthocyanins are glycosidated derivatives of the 3,5,7,3′-tetrahydroxyflavylium cation (Fig. 1). Glycosidation occurs at the 3, 5 and 7 positions. The non-glycosidated molecule (aglycone) is the anthocyanidin. Anthocyanidins rarely occur in nature and are usually found to occur as an artifact of the isolation process. The most common sugars found are monosaccharides such as glucose, galactose, arabinose and rhamnose. Di- and trisaccharides also occur. In some cases, the sugar moieties are acylated by *p*-coumaric, caffeic, ferulic or sinapic acids and sometimes by *p*-hydroxybenzoic, malonic or acetic acids. When present, these acyl substituents are usually bonded to the C-3 sugar [1].

The anthocyanins occur in plants at specific quantitative and qualitative distributions, hence they are very useful as a biochemical plant chemotaxonomic marker and as an index for quality control and quality assurance in fruit and vegetable products [2].

The objective of this article is to present a review of the chromatographic methods used to separate this class of important plant pigments.



	R_1	R_2
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Pelagonidin	H	H
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

Fig. 1. Structural formulas of the anthocyanins

2. ANALYSIS OF ANTHOCYANINS

2.1. Extraction

The anthocyanins are generally very soluble in water and can be easily extracted with polar solvents. Most workers employ acidified alcoholic solvents such as dilute (1%) HCl in methanol for extracting anthocyanins from plant materials [3,4]. Acidification is necessary to prevent oxidation, as anthocyanins are unstable at neutral and alkaline pH [5].

2.2 Sample clean-up

A number of methods have been succesfully used to clean-up crude anthocyanin extracts. They include solid-phase extraction with insoluble polyvinylpyrrolidone (PVP) [3,6–8], octadecylsilane [9–12], Sephadex G-25 [11], Sephadex LH-20 [11,12], polyamide [12,13], ion-exchange resins [4,14–18], acid alumina [19], precipitation with basic lead acetate [20,21] and solvent–solvent extraction with *n*-butanol [17,22]. The purification through solid-phase extraction is relatively simple. The procedure involves application of crude extracts of anthocyanins to a column or small, disposable cartridge of adsorbent and subsequent sequential elution of individual components with appropriate solvents. The anthocyanins possessing a number of unsubstituted hydroxyl groups (Fig. 1) or a sugar are strongly bound onto the adsorbents. Acidified methanol [6,7,9–12] is generally a suitable solvent for elution of anthocyanins from polymeric adsorbents.

2.3. Paper and thin-layer chromatography

Almost all of the pioneering work in the area of identification and characterization of anthocyanins has been performed with paper chromatography (PC). The literature is replete with information on solvent systems and R_F values for most of the known anthocyanins. Thin-layer chromatography (TLC) has been an attractive alternative to paper chromatography. Different stationary phases can be employed, thus opening up the possibility of many new separation mechanisms. TLC has the advantage in time over paper methods, but suffers from the disadvantage of low sample loading. Unfortunately, R_F values are not as reliable in TLC as with PC because of the differences in layer thickness

from plate to plate. In most cases, reference compounds are needed to confirm identification [23]. The paper methods are well entrenched and are unlikely to be replaced entirely by TLC methods [24].

There are a number of papers which give an excellent discussion of the uses of the various solvent systems which have been successfully used for separation of anthocyanins. The reader is referred to refs. 4, 18, 20 and 24–27 for a detailed discussion of the topic and PC and TLC.

2.4. Droplet counter-current chromatography

Counter-current chromatography (CCC) shares the same technologies as high-performance liquid chromatography (HPLC) such as pump, injector and detector except for different separation devices, columns. Although CCC could replace expensive, reversed-phase preparative-scale HPLC, it has not attracted a large number of users from industry and research involved in anthocyanin pigments. Francis and Andersen [28] described the use of droplet CCC (DCCC) for separation of the anthocyanins from black currants and raspberries using *n*-butanol, acetic acid and water as the solvent. These authors demonstrate that DCCC is a viable method for semi-preparative isolation of anthocyanins from berries.

2.5. Electrophoresis

The anthocyanins are ionic in nature (pH dependent) and would therefore be expected to be mobile in an electric field. Electrophoresis however, has found little use in the field of anthocyanin separations as it offers little or no advantage over PC. In acetate buffer, the anthocyanins do not migrate far. Ionization of the anthocyanins by alkali causes oxidative decomposition with air [23]. Successful results have been reported for paper electrophoresis using 0.1 *M* citric acid at pH 2 as an electrolyte [29]. Osawa *et al.* [30] obtained excellent results using cellulose acetate film. More recently, Tsuda and Fukuba [31] reported successful separation of anthocyanins by electrophoresis when using a Triton X-100/$AlCl_3$ containing electrolyte system.

2.6. Open column chromatography

In search of methods for larger-scale separation and quantitation of individual anthocyanins, column chromatographic methods have been developed. A number of column support materials have been tried without success, including aluminium oxide, cellulose powders, ion exchange resins and Sephadex gel. In most cases, sample matrix components were found to interfere with the separation. Polyamide powders showed good retention but chromatographic resolution was poor. The most success has been obtained with PVP [23].

2.7. Gas chromatography

Anthocyanins exhibit limited volatility and therefore require derivatization prior to gas chromatographic (GC) analysis. The most success has been achieved with reaction of the anthocyanins with trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS). The result is a nitrogen-containing derivative which after injection into the GC system further transforms into a quinoline derivative. The quinoline derivative yields sharp peaks when chromatographed [32]. Despite the excellent results obtained by previous workers using GC, derivatization in general introduces problems of stability in the anthocyanins and thus, future development is likely to be in the direction of HPLC rather than GC [24].

2.8. High-performance liquid chromatography

HPLC is the mainstay of the separation technique in food analysis, especially for water-soluble, non-volatile, thermally labile anthocyanins. The most success for separation of both anthocyanidins and anthocyanins has been with reversed-phase HPLC (RP-HPLC). This methodology offers excellent separations along with high sensitivity and relatively short analysis time (especially when compared with PC methods). HPLC with the combination of electrochemical detection (ED) and a UV–VIS or photodiode array detection (PAD) system makes structural characterization of the pigments possible [33]. The remainder of this paper focuses on the use of HPLC for separation of the individual anthocyanins in plant products (anthocyanin profile) and its application in food analysis.

3. ANTHOCYANIN PROFILE AND ITS APPLICATIONS IN FOODS

3.1. Applications in characterization of food anthocyanins

Anthocyanin pigments in many foods have been characterized by PC [1,4,7,17,18,34–45], TLC [6,7,

9,44,47–52] and more recently HPLC [2,3,9,12,13, 53–70]. Basically similar liquid chromatographic methods have been reported in the literature by many analysts and examples of HPLC methods that can be used for analysis of each of the anthocyanins from apple, bilberry, black currant, blackberry, black cherry, blueberry, blood orange, bog whortleberry, chicory, chokeberry, cowberry, crowberry, cranberry, elderberry, grape, grape colorants, lychee, raspberry, red currant, roselle, red wine, strawberry, European cranberry (*Vaccinium oxycoccus*) and *Vaccinium japonicum* fruits are summarized in Table 1.

In most cases, the reported systems were carried out on reversed-phase chromatography on silica-based ODS (C_{18} bonded phases) columns. The average particle diameter of HPLC packings is typically between 3 and 10 μm. HPLC analysis with columns of smaller particles (*e.g.* 3 μm) permit faster separations compared with columns of larger particles. However, 5–6-μm particles generally represent a good compromise in terms of convenience, performance and column lifetime. Also, silica columns bonded with octyl (C_8) and hexyl (C_6) were used in chicory [71], red wines [56], grape [58] and in bilberry fruits [72].

Gradient elution seems ideal for separating anthocyanins which are structurally very similar. Most of the solvent systems used in analytical HPLC include binary gradient elutions with methanol or acetonitrile as organic modifiers, and occasionally isocratic elution for fruits which have a relatively simple pigment pattern such as cranberry (Table 1). Ternary gradient elution was also developed for the separation of complex mixtures of anthocyanins in *V. rotundifolia* grapes [73]. Twenty-five anthocyanins including mono- and diglucosides of acylated and non-acylated anthocyanins in varying quantities were separated from *V. rotundifolia* hybrid grapes in 80 min. Because of the high viscosity of methanol–water mixture, acetonitrile was preferred [62]. Nagel and Wulf [74] substituted acetone for methanol to obtain a similar separation. A linear gradient using 5–20% acetone in place of the methanol separated 16 different anthocyanins from Cabernet Sauvignon [74]. Drdak *et al.* [68] determined that alkylamines as mobile phase additives and butylamine (0.122 *M*) in mobile phase with gradient elution provided better resolution and less retention of red wine anthocyanins. In particular, the nature of the organic modifier in the mobile phase had a dramatic influence on the separation of anthocyanin compounds. There was appreciable improvement in selectivity values for the separation of Cy-3-glurut, Cy-3-glc and Pg-3-soph (see Table 1 for abbreviations) as the organic modifier was changed from 100% methanol to 15% acetic acid in methanol [75].

The elution strength of mobile phase (water–acetonitrile–formic acid, 81:9:10) for chromatography of red fruits containing monoglycosylated anthocyanins such as bilberry, black currant, strawberry, blackberry, black cherry and morello cherry needed to be lowered to water–acetonitrile–formic acid (84:6:10) to improve the resolution of diholoside or triholoside anthocyanins from red currant, raspberry and elderberry [63].

Solvent systems for HPLC analysis of anthocyanins always include an acid to ensure that the anthocyanins are in the red flavylium cation form. Formic acid up to 10% (w/v) is most commonly used with reversed-phase columns which corresponds to pH about 1.9 [63]. Other acids such as acetic acid [57,73,75–80], phosphoric acid [9,11,54,81,82], trifluoroacetic acid [83] and perchloric acid [58,68,71] have also been used. However, extensive use of solvents more acidic than pH 2 could result in poor reproducibility and short column lifetime due to the loss of bonded phases from the surface of the silica stationary phase support. For this reason, non-silica polymer columns based on polystyrene were utilized [9,11,54,57,82]. Non-silica, polymeric columns, which are stable from pH 1 to 13, allowing their use with mobile phases at a pH close to which the anthocyanins are nearly entirely in their flavylium cation form, can produce sharp peaks.

Detection is usually carried out between 520 and 546 nm in the visible range. All the major anthocyanins have been identified based on acid hydrolysis, partial hydrolysis and subsequent determination of sugars and aglycone, spectral analysis, and co-chromatography with known available anthocyanins. Spectral acquisition was facilitated by applying on-line PAD for each peak eluting from HPLC columns [9,11,53,54]. Elucidation of structure, especially for the acylated anthocyanins, was further confirmed by fast atom bombardment mass spec-

trometry and NMR spectroscopy [12,58,71,84–87].

In an alternative method for the isolation of the anthocyanins of interest [2,58,88], a preparative HPLC system similar to that used for the analytical separation enabled isolation of pure samples of anthocyanins for use in structural studies. Hicks *et al.* [2] demonstrated that it was possible to collect multimilligram quantities of pure anthocyanins in one continuous 24-h period from blackberry and cranberry with preparative HPLC under binary gradient condition using a mobile phase consisting of 0.1 *M* phosphate buffer, pH 1.5 and acetonitrile. Sapers *et al.* [88] injected 250 μl of blackberry extract containing 15 mg of solid on Rainin Dynamax C_{18} preparative column to collect four anthocyanin peaks. Most of the semi-preparative HPLC columns are wide bore such as 10 mm [89], 16 mm [58,71] or 22 mm [2,88], and flow-rate was increased up to 14 ml/min [2].

For HPLC of anthocyanidins, isocratic conditions [3,9,63,75,78,90] are preferred because of the simplicity in commonly occurring anthocyanidins in nature and the retention of the aglycone moiety is correlated with the hydrophobicity of the molecule. RP-HPLC on a μBondapak C_{18} column (300 × 4.0 mm) with mobile phase of water–acetic acid–methanol (71:10:19) with 2 ml/min elution could resolve six anthocyanidins in 30 min [3]. Since anthocyanidins do not occur in the free form but are usually glycosylated, an acid hydrolysis step is required before analysis. As usual in RP-HPLC, the elution order is according to their polarity, delphinidin <cyanidin <petunidin <pelagonidin <peonidin <malvidin [3,63].

3.2. Applications for verification and classification of cultivars

In most of the work with anthocyanin pigmented fruits such as blueberries [43,48], raspberry [13,40,47,91], blackberry [91] and black grape [41,46], the distribution of individual anthocyanin pigments was determined by conventional techniques such as TLC and densitometry to delineate any quantitative or qualitative differences in cultivars. However, where a chemical marker is present in one plant and absent in the other, such qualitative differences make a positive identification easy. Unfortunately, at the cultivar level most of the differences found have been quantitative, and these conventional techniques can not always be easily adapted to quantitation. Especially with PC, quantitation must be regarded as estimated and relative because of the possible preferential anthocyanin binding to the paper chromatogram during development and elution and varying stability of the anthocyanin in the solvent systems used [43]. Furthermore, anthocyanins are not always completely separated. Cyanidin-3-glucoside is virtually impossible to separate from cyanidin-3-galactoside by column or TLC [64]. Also, although traces of other anthocyanins often were present, only major anthocyanins were identified.

For chemotaxonomy, HPLC techniques offer several advantages over TLC, especially to reveal the differences in relative percentages of individual anthocyanins and make it possible to distinguish relevant cultivars [64,92]. Applications of analytical HPLC in classification of some of fruit cultivars [58,73,75,80,93–95] are included in Table 1 and Fig. 2. Sapers *et al.* [94] developed the binary gradient elution with 15% acetic acid in water (solvent A) and 15% acetic acid in methanol (solvent B) to distinguish both qualitative and quantitative differences in anthocyanins between 11 relevant blueberry cultivars [94]. A rather complex gradient elution pattern was developed for 12 cranberry cultivars. A total of 22 peaks were separated in 60 min using the two C_{18} analytical columns (Nova-Pak and μBondapak C_{18} from Waters) connected in series and eluted with ternary solvent systems of aqueous formic acid, methanol and acetonitrile mixtures [95].

Besides the above applications, HPLC analysis of anthocyanin profile has also been used in red wines [56,58] for geographical classification. Wine color, in many cases, can be related to the quality and quantity of their anthocyanins. Recently, Santos *et al.* [96] used HPLC–PAD analysis of anthocyanin profiles and HJ biplot statistical analysis to differentiate 48 young red spanish wines according to their origin. The 18 anthocyanins were resolved by gradient elution (solvent A = 4.5% formic acid in water, solvent B = acetonitrile) with initial mobile phase composition of 10% B to a final composition of 30% in the same solvent system over a period of 37.5 min at a flow-rate of 1.5 ml/min. The non-acylated/acylated anthocyanin ratio was one of the best parameters to discriminate between the relevant origins.

TABLE 1

COMPILATION OF REFERENCES ON THE SEPARATION OF ANTHOCYANINS BY RP-HPLC

Abbreviations: ac = acetyl; ara = arabinoside; caf = caffeyl; cm = coumaryl; Cy = cyanidin; Dp = delphinidin; fer = ferulyl; gal = galactoside; glc = glucoside; glcrut = glucosylrutinoside; mal = malonyl; Mv = malvidin; Pg = pelagonidin; Pn = peonidin; Pt = petunidin; rut = rutinoside; samb = sambubioside; sin = sinapyl; soph = sophoroside; xylrut = xylosylrutinoside.

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Apple	Cy-gal,ara	μBondapak C_{18}	Water–acetic acid–methanol (71:10:19)	76
Bilberry	Dp,Cy-gal,glc Pt-gal,glc Pn-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	Aquapore RP-300, C_8	Binary gradient (A) 10% formic acid (B) methanol–acetonitrile–water–formic acid (22.5:22.5:45:10)	72
Black currant	Dp,Cy-glc,rut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Dp,Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Cy,Dp-glc,rut	Spherisorb ODS	Binary gradient (A) 0.5% trifluoroacetic acid (B) methanol	83
Blackberry	Cy-glc,rut,soph Cly-glcrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
	Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Cy-glc,rut	Resolve C_{18}	Binary gradient (A) 0.1 *M* potassium dihydrogenphosphate (B) acetonitrile	88
Blueberry	Cy-gal,glc,ara Dp-gal,glc,ara Pt-gal,glc,ara Pg-gal,glc,ara Mv-gal,glc,ara	HS-5 C_{18}	Binary gradient (A) 10% formic acid (B) acetonitrile	93
	Cy-gal,glc,ara Dp-gal,glc,ara Pt-gal,glc,ara Pg-gal,glc,ara Mv-gal,glc,ara	μBondapak C_{18}	Binary gradient (A) 0.1 *M* potassium dihydrogenphosphate (B) acetonitrile	94
Bog whortle berry	Dp-gal,glc,ara Cy-gal,glc,ara Pt-gal,glc,ara Pn-gal,glc,ara Mv-gal,glc,ara	Supelcosil LC-18	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (10:40:50)	66

TABLE 1 (*continued*)

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Cherry	Cy-glc,rut	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
	Cy-glc,rut Cy-soph,glcrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Chicory	Cy-glc-mal	Spherisorb C_6	Binary gradient (A) 0.6% perchloric acid (B) methanol	71
Chokeberry	Cy-gal,glc,ara,xyl	Spheri-5 RP-18	Binary gradient (A) 10% formic acid (B) formic acid–water–acetonitrile (10:60:30)	69
Cranberry *(V. macrocarpon)*	Cy-gal,ara Pn-gal,ara	Resolve C_{18}	Binary gradient (A) 0.1 *M* potassium dihydrogenphosphate (B) acetonitrile	2
	Cy-gal,glc,ara Pn-gal,glc,ara	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
	Cy-gal,ara Pn-gal,ara	μBondapak C_{18}	Water–acetonitrile–acetic acid–orthophosphoric acid (81.7:8.4:8.4:1.5)	80
	Cy-gal,ara Pn-gal,ara Cy-glc	PLRP-S	Binary gradient (A) 10% acetic acid (B) methanol–acetic acid–water (60:10:30)	57
	Cy-gal,ara Pn-gal,ara	μBondapak and Nova-Pak C_{18}	Ternary gradient (A) 4.5% formic acid (B) methanol–formic acid–acetonitrile (55:33:10) (C) methanol–formic acid–acetonitrile (55:35:10)	95
Cranberry *(V. oxycoccus)*	Cy-gal,glc,ara Pn-gal,glc,ara Dp,Pt,Mv-glc	Supelcosil LC-18	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (1:4:5)	125
Cowberry	Dp-glc Cy-gal,glc,ara	Hypersil ODS	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (10:40:50)	67
Crowberry	Dp,Pt-gal,glc Cy,Pn,Mv-gal,ara Dp,Pt-ara	μBondapak C_{18}	Water–methanol–formic acid (74:16:10)	111
Elderberry	Dp-gal,glc,rut Cy-glcrut	LiChrospher RP-18	Water–acetonitrile–formic acid (84:6:10)	63
	Cy-glc,samb Cy-sambglc Cy-diglc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54

(Continued on p. 228)

TABLE I (*continued*)

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Grape (*V. rotundi folia*)	Cy,Pn,Dp-glc Pt,Mv-glc Mv-glc-ac Cy,Pn,Pt,Mv-glc-cm Cy,Pn,Dp,Pt,Mv-diglc Dp,Cy,Pn,Pt,Mv-glc-cm-glc	LiChrosorb RP-18	Ternary gradient (A) 15% acetic acid (B) water–acetic acid–methanol (65:15:10) (C) methanol	73
	Dp,Cy,Pt,Pn,Mv-diglc Dp-glc	HS-5 C_{18}	Binary gradient (A) 10% formic acid (B) acetone–formic acid–water (25:10:65)	65
Grape (*V. vinifera*)	Dp,Cy,Pn-glc Pt,Mv-glc Mv-glc-ac Cy,Pn,Pt,Mv-glc-cm Cy,Pn,Dp,Pt,Mv-diglc Dp,Cy,Pn,Pt,Mv-glc-cm-glc	Spherisorb C_6	Binary gradient (A) 0.6% perchloric acid (B) methanol	58
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-diglc Dp,Cy,Pt,Pn,Mv-glc-cm Dp,Cy,Pt,Pn,Mv-glc-cm-glc	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid–methanol (65:15:20)	61
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Cy,Mv,Pt,Pn-glc-cm Mv-glc-caf	LiChrosorb ODS	Binary gradient (A) 10% formic acid (B) formic acid–methanol–water (10:50:40)	13
	Cy,Pn,Dp-glc Pt,Mv-glc Cy,Dp,Pt-glc-cm Pn,Mv-glc-cm	Spherisorb ODS	Binary gradient (A) 3.5% orthophosphoric acid (B) acetonitrile	81
	Dp,Cy,Pt-glc Dp,Cy,Pt-glc-ac Pn,Mv-glc-ac Dp,Cy,Pt-glc-cm Pn,Mv-glc-cm Pn,Mv-glc-caf	μBondapak C_{18}	Binary gradient (A) 4.5% formic acid (B) acetonitrile	53
Grape skin extract (Spreda)	Dp,Cy,Pt-glc Pn,Mv-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Grape colorants (Welch)	Dp,Cy,Pt-glc Pn,Mv-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Enocyanin (Minot)	Dp,Cy,Pt-glc	PLRP-S	Binary gradient (A) 10% acetic acid (B) methanol–acetic acid–water (60:10:30)	57
Plum	Cy-glc,rut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54
Raspberry (black)	Cy-samb,glc,rut Cy-xylrut	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	54

TABLE 1 (*continued*)

Source	Anthocyanins	Stationary phase	Mobile phase	Ref.
Raspberry (red)	Cy,Pg-glc Cy,Pg-soph Cy,Pg-glcrut	Supelcosil LC-18	Binary gradient (A) 15% acetic acid (B) 15% acetic acid in methanol	75
	Dp-gal,glc,rut Cy-glcrut Cy-gal	LiChrospher RP-18	Water–acetonitrile–formic acid (84:6:10)	63
	Cy-glc,rut Cy,Pg-soph Cy,Pg-glcrut	Supelcosil LC-18	Binary gradient (A) 15% acetic acid (B) acetonitrile	77
Red currant	DP-gal,glc,rut,ara Cy-gal,glc,rut,ara Cy-glcrut Pt,Pg-gal	LiChrospher RP-18	Water–acetonitrile–formic acid (84:6:10)	
Roselle	Cy,Dp-samb Dp,Cy-glc	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
Strawberry	Cy,Pg-glc Pg-rut Pg-glc-ac	PLRP-S	Binary gradient (A) 4% orthophosphoric acid (B) acetonitrile	9
	Cy-glc Pg-gal,glc,ara	LiChrospher RP-18	Water–acetonitrile–formic acid (81:9:10)	63
Red wines	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Cy,Pt,Pn,Mv-glc-cm	MicroPak MCH-5C_{18}	Binary gradient (A) 5% formic acid (B) methanol	126
	Dp,Cy,Pt,Pn,Mv-glc Dp,Cy,Pt,Pn,Mv-glc-ac Dp,Pn,Mv-glc-cm	CGX C_{18}	Binary gradient (A) 10% methanol–perchloric acid 0.16 *M*–butylamine 0.122 *M* (B) 90% methanol–perchloric acid 0.16 *M*–butylamine 0.122 *M*	68
	Cy,Dp,Pt-glc Pn,Mv-glc Mv-glc-cm Mv-glc-ac	LiChrosorb ODS	Binary gradient (A) 10% formic acid (B) acetone–formic acid–water (25:10:65)	74
Lychee	Cy-glc Cy-rut Mv-glc-ac	PLRP-S	Binary gradient (A) 3.5% orthophosphoric acid (B) acetonitrile	11
Blood orange	Dp,Cy,Pn-diglc Dp,Cy-glc Cy-glc-ac Cy-glc-fer Cy-glc-cmfer Cy-glc-sin Pn-glc-cm	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid–methanol (65:15:20)	79
	Dp,Cy,Pt,-diglc Pg,Pn-diglc Dp,Cy, Pg, Pt-glc Cy-glc-ac	μBondapak C_{18}	Binary gradient (A) 15% acetic acid (B) water–acetic acid–methanol (65:15:20)	78
V. japonicum	Cy,Pg-ara Cy,Pg-gal	Supelcosil C_{18}	Binary gradient (A) 10% formic acid (B) formic acid–water–methanol (1:4:5)	89

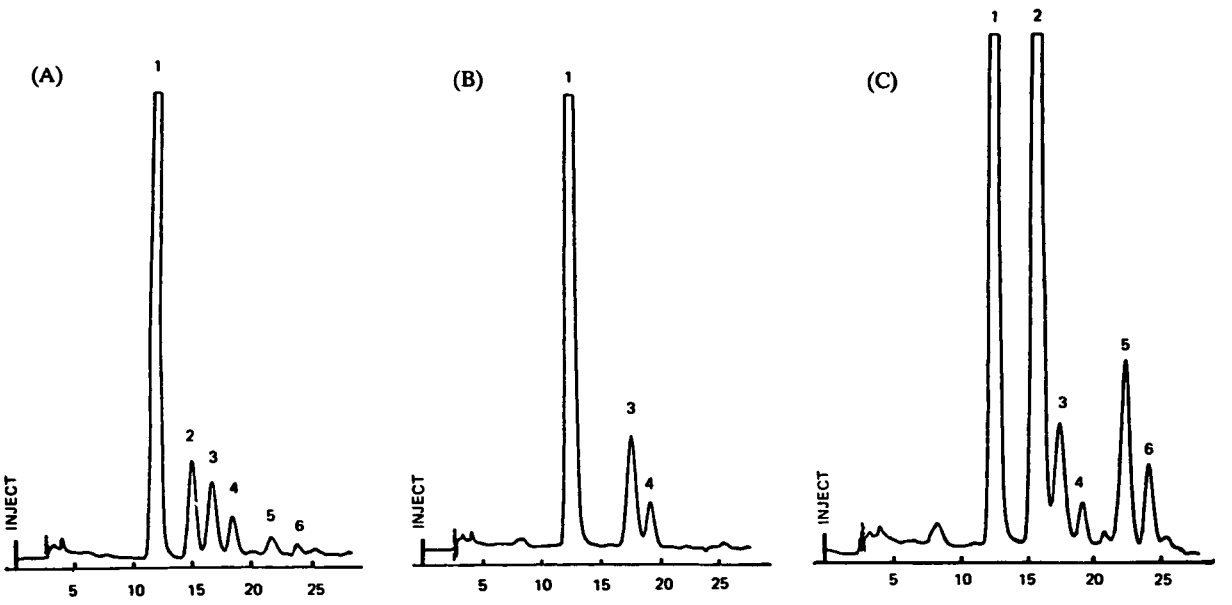


Fig. 2. HPLC of anthocyanins from red raspberries: (A) Meeker variety (Oregon); (B) Willamette variety (Oregon); (C) Marcy variety (New Zealand). Peaks (For abbreviations see Table 1): 1 = Cy-3-soph; 2 = Cy-3-glcrut; 3 = Cy-3-glc; 4 = Pg-3-soph; 5 = Cy-3-rut; 6 = Pg-3-glcrut. (Adapted with permission from ref. 75). Time scale in min.

3.3. Applications for changes in anthocyanins during ripening, processing and storage of fruit products

Very significant changes such as the accumulation of anthocyanins take place during the maturation of numerous red fruits. Quantitative determination of individual anthocyanins gave a great insight into the development of anthocyanins in the maturing red tart cherry [97], thornless blackberry fruits [88] and red grape [81]. Dekazos [97] reported the use of AG 50W-X4 (H^+ form), a cation-exchange resin, to purify cherry anthocyanins. Most of free sugars and organic residues were removed by washing with water, and methanol, respectively. Anthocyanin pigments were eluted by acidified methanol (0.1% to 1% HCl in methanol) and PC. There have been a few applications of HPLC in the investigation to determine the effects of ripening on the accumulation of individual anthocyanins. Sapers *et al.* [88] used binary gradient elution on a Resolve 5-μm C_{18} column using 0.1 *M* potassium dihydrogenphosphate (pH 2.0) and concave (program No. 7 of the Waters solvent programmer) gradient elution from 12% to 20% acetonitrile in 25 min to determine the effect of ripening on anthocyanin patterns in thornless blackberry fruit.

Anthocyanins are very reactive and lack stability during processing and storage. The rapid degradation of the attractive red color of freshly made blackcurrant juices [83], strawberry preserves [98], cranberry cocktail [99], canned plums [100] and red raspberry juices [77] during storage has been a concern to food processors. Numerous workers have applied chromatographic analysis to estimate the losses of red anthocyanin pigments during processing and storage of fruits [76,82,101–104], and during fermentation and subsequent aging periods of red wines [74,77]. Also, applications which have been extended to evaluate many factors such as temperature, light, pH, mold, 5-hydroxymethylfurfural, furfural and sugar on anthocyanin stability [53,105–110] and to compare the influences of structural variations of the different anthocyanins on their relative rate of reactivity [90,111–115] with other component have been reported. The pH differential method was readily applicable to the studies of determination of anthocyanin pigments [90, 98,105,108–110,114] during processing and storage. Although the method is not specific for individual pigments, it provides quantitative estimations of the total concentration of pigments, which reflects the

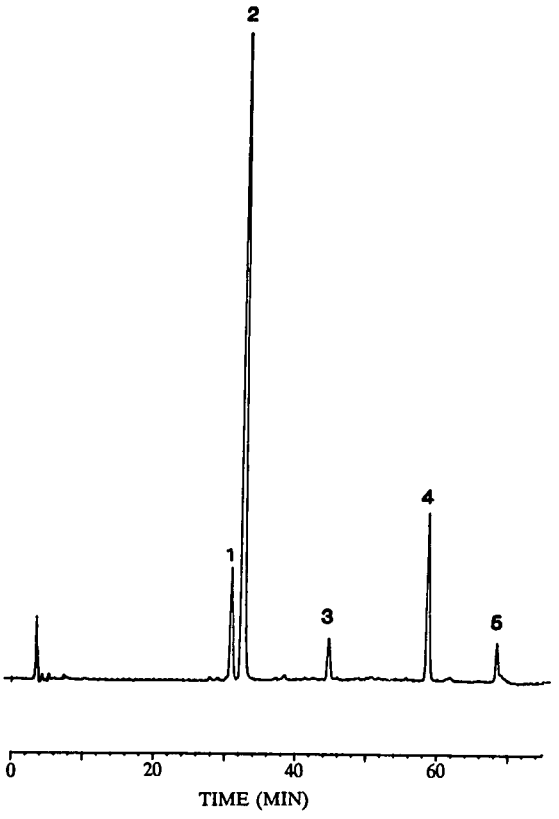


Fig. 3. HPLC of anthocyanins from lychee. Peaks: 1 = Cy-3-glc; 2 = Cy-3-rut; 3 = cyanidin; 4 = Mv-3-glc-ac; 5 = unknown. (Adapted from ref. 11).

quality of anthocyanin-containing food products.

Typical conditions for HPLC applications for anthocyanin pigment stability in manufactured fruit products [77,83] and red wines [74] are summarized in Table 1. Binary gradient HPLC analysis (Fig. 3) was applied for the quantitative determination of changes in Cy-3-glc, Cy-3-rut and Mv-3-glc-ac contents in tropical lychee fruit [11,82] during refrigerated storage conditions.

3.4. Applications for detection of adulteration of fruit juices

The distinctive anthocyanin pigment profile has been a useful tool for the verification of authenticity in fruit juice products which are rich in anthocyanin pigments, [116,117], and detecting the adulteration of red wines of *V. vinifera* with wines made from hybrid grapes [118]. For example, red raspberry has been reported to have only cyanidin and pelagonidin glycosides [75]. If chromatographic analysis reveals the presence of malvidin, delphinidin, peonidin or petunidin glycosides from commercial raspberry juice samples, it can be used to confirm and pinpoint adulteration with other colored fruits.

PC of anthocyanin pigment has been used to detect adulteration of concord grape juice with other anthocyanin containing products [119,120]. This method has been applied to several dark colored fruit juices, such as blackberry, black raspberry and black cherry juices to detect adulteration with cheaper juices based on anthocyanin or anthocyanidin patterns [121]. A similar approach was used recently by Kaack [122] for fruit juice adulteration. In his procedure, semi-quantitative direct spectrophotometric determination of paper strip at 530 nm was employed to resolve the problem of visual examination from paper chromatograms, which might not see the dinstinct differences in quantity.

Analysis of anthocyanins by HPLC proved to be easier to interpret than the qualitative patterns in paper or thin layer chromatography in determination of juice adulteration. Most of the reported work has been done with cranberry juice products since cranberry has a unique pattern of anthocyanins [116] and is relatively expensive, making economic incentives for adulteration attractive [57]. Fast HPLC separation using a 5 cm long analytical column was adopted for the verification of cranberry juice cocktail [116]. Analysis was completed in about 15 min by RP-HPLC using a mobile phase of water–acetonitrile–acetic acid–orthophosphoric acid (81.7:8.4:8.4:1.5) on a Supelcosil C_{18} column (5 μm). Two major anthocyanins such as cyanidin-3-galactoside and peonidin-3-galactoside, and two minor anthocyanins of cyanidin-3-arabinoside and peonidin-3-arabinoside were well separated.

For more simple chromatographic analysis, HPLC anthocyanidin analysis of cranberry juice products, which requires acid hydrolysis of anthocyanin glycosides in sample preparation, was elected [123]. Only cyanidin (57%) and peonidin (43%) were found in the cranberry juice samples [124]. Fig. 4 presents two representative anthocyanidin chromatograms showing separations of authentic cranberry juice (Fig. 4A) and adulterated juice (Fig. 4B). Mobile phase used was water–acetic acid–methanol–acetonitrile (70:10:10:10). The presence of sub-

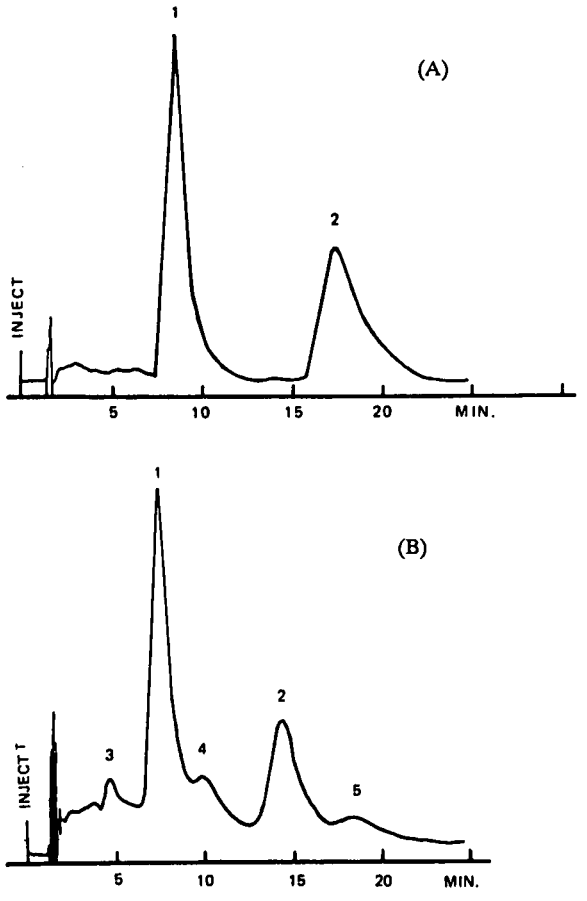


Fig. 4. HPLC of anthocyanins from cranberry juices. (A) Authentic cranberry juice; (B) adulterated commercial cranberry juice drink. Peaks: 1 = cyanidin; 2 = peonidin; 3 = delphinidin; 4 = petunidin; 5 = malvidin. (Adapted from ref. 124).

stantial quantities of delphinidin, petunidin and malvidin in the commercial samples reveals that an anthocyanin-containing colorant has been added. RP-HPLC analysis using acetic acid–water (10:90) (A) and methanol–acetic acid–water (60:10:30) (B) gradient with gradient time of t_G = 30 min was also applied to detect adulteration of cranberry juice by enocyanin, a grape skin colorant made usually as a by-product of wine making [57]. Reversed-phase separation was carried out with a C_{18} column made from polystyrene (PLRP-S, 150 × 4.6 mm from Polymer Lab.). The pH of the mobile phase was maintained at 1.3 by the addition of 3% phosphoric acid.

4. CONCLUSIONS

This article presents a brief review of analysis of anthocyanins as well as applications in food analyses. Discussion was focused on the use of HPLC for separation of individual anthocyanins. No attempt was made to give a coverage of the literature relating to anthocyanin pigments. The advent of a highly selective and sensitive mode of detection using HPLC for easy structural identification of anthocyanins where standards generally are not available will undoubtly lead to further advances in characterization of anthocyanins in fruits and berries.

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Review

Chromatographic analysis of *cis/trans* carotenoid isomers☆

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ABSTRACT

Cis/trans configurations of carotenoids are known to effect the biochemistry of carotenoids in certain situations. Methodology for separating carotenoid *cis/trans* isomers is of importance to nutritionists and food scientists because *cis* isomers of provitamin A carotenoids have lower provitamin A activities than the all-*trans* form. Traditional food processing and preservation methods, especially thermal treatments, induce the formation of *cis* isomeric forms. However, many challenges are apparent for identifying and analyzing *cis/trans* isomers present in foods and other biological tissues. The development of current chromatographic methods for the separation of carotenoid *cis/trans* isomers is reviewed. For the separation of β-carotene isomers, most procedures employ either $Ca(OH)_2$ or Vydac C_{18} columns. In general, polymeric C_{18} columns allow for the detection of *cis* carotenes, while monomeric C_{18} columns provide for some separation of certain xanthophylls. The main *cis* isomers detected in foods are the 13-*cis* and 9-*cis* forms, although other forms have also been found (mainly 15-*cis* and various di-*cis* isomers). More studies involving the metabolism and physiological consequences of *cis/trans* isomers in the diet are needed. However, due to limitations in current techniques, further method development in the area of separation, detection and quantitation of *cis/trans* carotenoid isomers will be required.

CONTENTS

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1. INTRODUCTION

Carotenoids have many biochemical roles, including the important functions of light harvesting and photoprotection in photosynthesis [1,2] as well as the provitamin A activity of certain carotenoids [3]. There is growing evidence that carotenoids may have a protective effect against the development of certain cancers [4], perhaps due to their antioxidant and free radical quenching activity [5]. In addition, some evidence suggests that carotenoids may have an immune enhancement effect that helps to limit cancer growth [6].

Currently, 563 naturally occurring carotenoids have been isolated and identified [7]. Individual carotenoids are capable of forming different *cis/trans* geometrical isomers. Geometrical configuration is known to effect the biochemistry of carotenoids in certain situations. For example, carotenoids exist in the 15-*cis* form in the reaction centers and the all-*trans* configuration in the light harvesting centers of photosynthetic organisms [1]. Animal studies indicate differences in provitamin activity among the *cis/trans* isomers of provitamin A carotenoids [8]. Isomer forms of carotenoids may have physiological or metabolic roles that are still being investigated. Also, methodology for the separation of *cis/trans* carotenoid isomers is necessary for accurate determination of provitamin A activity in foods. Analyses of processing effects on carotenoid content also require separation of the various *cis/trans* isomers. While many review articles on the analysis of carotenoids have been written [9–11], few have discussed recent studies concerning the various isomers of certain carotenoids. A comprehensive text on *cis/trans* isomers of carotenoids and polyenes was written in 1962 by Zechmeister [12]. More recent reports are scattered throughout the literature.

The purpose of this paper is to present a thorough review of the chromatographic methods used to separate carotenoid *cis/trans* isomers and to discuss the results of the work for which these methods were developed. Also, included are discussions on the identity of the various isomers being detected through the application of new chromatographic methods. Furthermore, information is presented on the chemistry, biochemistry and/or nutritional implications of these isomers. An overview of the extraction and separation procedures for carotenoids in general is included along with some of the historical developments which contributed to our current understanding of carotenoid *cis/trans* isomers.

2. CAROTENOIDS AND THEIR *CIS/TRANS* ISOMERS

2.1. Nomenclature

Carotenoids are composed of isoprene units linked to form a conjugated double bond system. The pigmentation of these compounds is the result of the chromophore created by the series of conjugated double bonds. Typically, carotenoids contain eight isoprenoid units bonded such that the units are reversed at the center of the molecule. With this arrangement, many carotenoids are symmetrical in nature. The two central methyl groups are arranged in a 1,6 position relative to each other with the remaining nonterminal methyl groups forming a 1,5 positional relationship [13]. The structure of lycopene, shown in Fig. 1, illustrates a typical example.

The basic acyclic structure of joined isoprene units can be modified by hydrogenation, dehydrogenation, cyclization or oxidation [14]. The oxygenated derivatives are known as xanthophylls, while the hydrocarbon carotenoids are referred to as carotenes. The nomenclature of carotenoids is based on the carotene backbone and because of the symmetrical structure, one half of the molecule is numbered 1–15 from the end to the center, with addi-

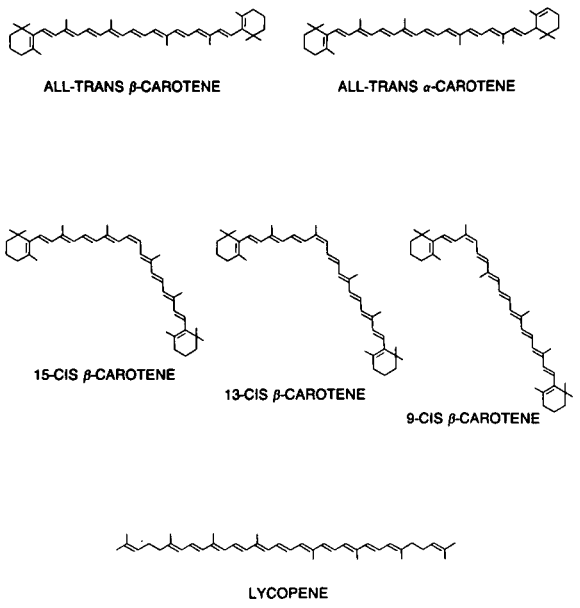

Fig. 1. Chemical structures of several carotenes.

tional methyl groups numbered 16–20. The other half is numbered in the same manner, only 1′–15′ and 16′–20′. Xanthophylls are widespread in nature, including hydroxy, methoxy, glycosyloxy, and carboxy derivatives [13] and these are often found esterified [15]. β-Carotene is a carotene which has undergone cyclization on either end to form two β-cyclohexene end-groups. β-Carotene is an example of a symmetrical carotenoid, while α-carotene is not, because it has a β-cyclohexene group on one end of the molecule and ε-cyclohexene group on the other.

Carotenoids undergo geometrical changes about the double bonds. Using the IUPAC nomenclature rules of carotenoids approved in 1974 [16], the stem name implies *trans*, thus *cis* configurations are specified by citing the specific bond as being *cis*. In Fig. 1, the β-carotene isomers are labeled with this terminology. These nomenclature rules will be used in this manuscript, instead of designating double bonds as *E* or *Z*. Isomers known to have at least one *cis* double bond but that are of uncertain configuration will be referred to as a *cis* isomer. The prefix "*neo*" has also been used to designate *cis* isomers of unknown configurations [17].

2.2. Historical background

While the existence of carotenes and xanthophylls was known prior to 1906, it was not until Tswett [18] developed column chromatography that much was known about the carotenoids. After its introduction by Tswett, open column chromatography became the principle technique for the separation of carotenoids. After Gillam and El Ridi [19] observed β-carotene separated into two zones with repeated adsorptions on an alumina column, investigations on the separation of geometrical isomers by column chromatography began. Polgár and Zechmeister [20] separated three major β-carotene bands, using a calcium hydroxide [$Ca(OH)_2$] packed column and a developer of petroleum ether with 1–5% acetone. Zechmeister [12] developed a nomenclature system to distinguish *cis* isomers of the same carotenoid separated chromatographically, but of unknown configuration. The *cis* isomers eluting prior to the all-*trans* carotenoid were referred to as *neo*-A, *neo*-B, *etc.*, while those isomers eluting after the all-*trans* compound are referred to as *neo*-U, *neo*-V, *etc.* [12]. The two main *cis* isomer bands for β-carotene were referred to as *neo*-β-carotene B and *neo*-β-carotene U by Polgár and Zechmeister [20]. Bickoff *et al.* [21] worked on improving the separation of these isomers, and found better separation with a developer of 1.5% *p*-methylanisole in petroleum ether.

Several treatments have been found that resulted in the formation of an equilibrium mixture of *cis*–*trans* isomers. These include: refluxing in organic solvents, melting of crystals, contact for prolonged periods with certain active surfaces, treatment with acids, and illumination of solutions (catalyzed by iodine) [12,14]. The equilibrium mixtures of isomerized carotenoids generally show two or three predominant *cis* isomers, which are referred to as the main or preferred isomers [12,14].

While the results of chromatographic separation revealed only several isomers, theoretically large numbers of possible geometrical configurations could occur because of the large number of double bonds in carotenoids (272 such isomers for β-carotene) [14]. The theory that best explains the limited number of preferred isomers was proposed by Pauling [22] in which two types of double bonds were described (hindered and unhindered). The methyl-substituted double bonds and the central double

bond in C_{40} carotenoids are generally unhindered from the formation of a *cis* configuration. The remaining double bonds are hindered, because the formation of the *cis* configuration is restricted by steric hindrance encountered between the methyl group attached to one of the adjacent carbons and a hydrogen attached to the other adjacent carbon. For β-carotene, only five double bonds are unhindered (position 9, 9', 13, 13', and 15–15') [12]. Symmetrical carotenoids such as β-carotene have fewer possible configurations, because the formation of a *cis* double bond in the same position on either half of the molecule would yield the same structure; for example 9-*cis*-β-carotene and 9'-*cis*-β-carotene are the same molecule. Of the possible 20 configurations of β-carotene formed by *cis* isomerization about unhindered bonds, open column chromatography revealed 12 *cis* isomers with only two considered main isomers, *neo*-U and *neo*-B [12].

Spectroscopic properties were used by early chromatographers to distinguish between pigments and as a means to gain insight into possible structures. Absorption spectroscopy is still the major technique to identify and quantitate carotenoids [13]. The color of a carotenoid is produced by its absorption in the 400–500 nm region of the visible spectrum. Generally there are three maxima or two maxima and a shoulder, with the middle peak having the highest intensity [12]. One of the main determinants for the absorption spectra of these compounds is the number of conjugated double bonds. Longer chromophores have absorption maximum at longer wavelengths [13;14]. Modification from the basic acyclic structure can lead to wavelength shifts, such as shifts to longer wavelengths when carbonyl groups are added to the structure, or shifts to shorted wavelengths with the formation of ring structures [14]. The amount of fine structure in the spectral curves' shape and the intensity of absorption also vary among the carotenoids.

The absorption spectra of carotenoids are affected by the sample solvent. However, for a given solvent, the absorption spectra of an unknown carotenoid can be compared to spectra of known carotenoids to aid in identification. Reported absorptivities for given compounds are useful for the quantitation of the carotenoids. For example, the reported $E^{1\%} = 2592$ for β-carotene at 453 nm can be used to determine the amount of all-*trans*-β-carotene in a hexane solution [23].

As was found with different carotenoids, geometrical isomers of the same compound have different absorption spectra. In the visible region, the maxima shift to shorter wavelengths and the degree of fine structure decrease for spectra of *cis* isomers as compared to spectra of the compound in the all-*trans* configuration [12]. In the UV region, an absorption peak referred to as the *cis* peak is apparent for *cis* isomers, with the intensity of this peak varying with the position and number of *cis* double bonds [12]. For the mono-*cis* isomers of β-carotene, the intensity of the *cis* peak increases when the *cis* bond is located closer to the center of the molecule. Thus, 13-*cis*-β-carotene has a higher absorptivity in the *cis* peak region than does 9-*cis*-β-carotene. The UV–visible absorption spectra for β-carotene isomers, shown in Fig. 2, demonstrate the characteristic differences in absorption for carotenoid *cis*/*trans* isomers.

2.3. *Provitamin A activity of carotenoids*

While the importance of maintaining adequate levels of vitamin A in the body has long been known, in many parts of the world both clinical and "subclinical" vitamin A deficiencies occur [24]. It has been estimated that 500 000 new cases of corneal xerophthalmia occur yearly in parts of Asia [25]. In addition to these cases of individuals showing clinical signs of vitamin A deficiencies, studies

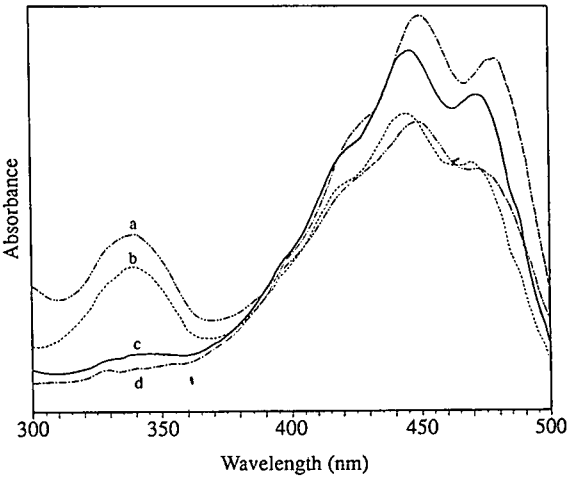

Fig. 2. Absorption spectra of (a) 15-*cis*-β-carotene, (b) 13-*cis*-β-carotene, (c) 9-*cis*-β-carotene and (d) all-*trans*-β-carotene. From O'Neil *et al.* [78].

have shown that vitamin A supplementation with children in Indonesia, India and South Africa can reduce mortality rates even in children without active xerophthalmia [26–28]. Because animal products are typically not accessible to those at risk for deficiency, provitamin A carotenoids derived from plant tissues are important in maintaining vitamin A levels.

Since it can be cleaved to theoretically yield two molecules of retinal, β-carotene has the highest provitamin A activity of the carotenoids. However, feeding studies suggest that the *cis* isomers of β-carotene have lower provitamin A activities when compared to the all-*trans* form [8,29–31]. Sweeney and Marsh [8] performed a vitamin A liver storage study of rats fed various carotene isomers. The rats fed *cis* isomers of β-carotene were found to have as low as 61% of the vitamin A liver storage compared to the amount of vitamin A stored by rats fed all-*trans*-β-carotene.

Most studies have shown that other provitamin A carotenoids have similar decreased vitamin A activity in the *cis* form. Sweeney and Marsh [8] found *cis* isomers of α-carotene also had lower vitamin A liver storage values in rats than all-*trans*-α-carotene. *Cis* isomers of cryptoxanthin and most *cis* isomers of γ-carotene have been shown to have lower provitamin A activity [32]. One exception is pro-γ-carotene, a penta-*cis* isomer of γ-carotene which was found to have a higher provitamin A activity in rats in comparison to all-*trans*-γ-carotene [33].

2.4. Extraction and separation of carotenoids

The conjugated double bond systems of carotenoids causes them to be particularly unstable compounds, especially sensitive to light, heat, oxygen, and acids [14]. Because of this instability, several precautions are necessary, when handling carotenoids. For example, laboratory experiments should be carried out in dim lighting, evaporation should be performed by rotary evaporation at reduced pressures with final stages of evaporation carried out under a stream of nitrogen, and samples should be stored in the dark, under nitrogen, at about −20°C [14].

The extraction procedures for carotenoids vary because of the wide variety of tissues and products containing these compounds. Detailed description of extraction procedures for various products have been discussed [14,34]. Carotenoids are fat soluble but because of the high moisture content of plant tissues, a preliminary extraction solvent miscible with water is generally necessary to allow for penetration of the solvent. Methanol is often used as an initial extractant [14,34]. Water-immiscible solvents can be used after the sample is dehydrated, however it has been found that more efficient extractants are composed of a slightly polar solvent in addition to the nonpolar solvent [34]. After extraction, the carotenoids are transferred to the nonpolar solvent by separating the liquid phases; adding water can facilitate this separation [34]. Saponification of carotenoid extracts may be necessary to remove neutral fats, chlorophylls, and chlorophyll derivatives. This procedure can be accomplished by exposure to potassium hydroxide saturated methanol [34].

Chromatographic methods are still used for the separation of carotenoids, however, newer methods have generally replaced open column methods. Taylor [35], Lambert *et al.* [36] and Tee and Lim [11] have written review articles on the various chromatographic methods for the analysis of carotenoids and retinoids. Thin-layer chromatography (TLC) has been found to be useful for qualitative analyses and as an aid in identification based on adsorption affinities [14]. Zakaria *et al.* [37] reviewed a wide variety of applications for TLC, including pigments. High-performance liquid chromatography (HPLC) offers several advantages over the TLC and open column methods for both analytical and preparative separations. Rüedi [38] discusses the usefulness of HPLC technologies for the analysis of carotenoids. Rodriquez-Amaya [10] has critically reviewed and compared open column and HPLC methods for the analysis of provitamin A carotenoids from plant tissues.

Many researchers have developed and used HPLC methods to qualitatively and quantitatively analyze carotenoids. Thompson and Maxwell [39] developed reversed-phase methods to determine β-carotene in several dairy products. Zakaria *et al.* [40] developed a reversed-phase method to separate carotenes in tomatoes. Similar reversed-phase methods have been used with other fruits and vegetables [41–44]. The US Department of Agriculture Human Nutrient Composition Laboratory has published extensively in the area of HPLC separation methods for carotenoids and carotenoid fatty

acid esters [45–48]. Beecher and Khachik [49], discussing the analysis of carotenoids in foods using HPLC methods, summarized much of this laboratory's developments in this area.

A more recent review by these authors surveyed the current literature on the separation, identification and quantification of carotenoids, which included developments in the analysis of human plasma as well as plant tissues [50]. Khachik *et al.* have since developed HPLC methods for the separation of plasma carotenoids [51]. Others researchers have also developed methods to quantitate specific carotenoids in serum and plasma [52–56].

3. ANALYSES OF β-CAROTENE *CIS/TRANS* ISOMERS

3.1. Historical background

After the separation of β-carotene isomers by open column chromatography methods [20,21], *cis* isomers were detected in extracts from several food products [57–59]. An analysis of a wide variety of both fresh and processed products was performed by Panalaks and Murray [60], using open column chromatographic methods. These authors found only small amounts of *neo*-β-carotene B and *neo*-β-carotene U in the extracts from most of the fresh and treated products, but increases in the relative amounts of *cis* isomers, especially *neo* B, were found for some canned food products.

While many TLC methods have been developed for the analysis of carotenoids [34,36], there are few reports on resolving β-carotene isomers. Sadowski and Wójcik [61] detected three *cis* isomers for both α- and β-carotene from TLC separations of bean leaf extracts. The method involved TLC plates of magnesium oxide that were developed with solutions of acetone in light petroleum. Schwartz and Patroni-Killam [62] developed a TLC method based on open column chromatography methods, that resolved three β-carotene isomers and four α-carotene isomers. The TLC plates were of $Ca(OH)_2$ and these were developed with 1.5% *p*-methylanisole in petroleum ether. *Cis* isomers were detected in a number of carotenoid extracts from fresh and processed vegetables, with the exceptions of fresh sweet potato and carrot extracts.

3.2. Development of modern HPLC methods and their use in plant tissue analyses: normal phase

Pressurized liquid chromatographic separations of β-carotene isomers began with the work of Sweeney and Marsh [63]. Based on open column adsorption chromatography, a 30 cm × 15 mm column was packed with a mixture of magnesium hydroxide and $Ca(OH)_2$ and an eluent of 1.5% *p*-methylanisole in petroleum ether was pumped through by nitrogen pressure. The principle isomers detected in extracts from green vegetables were all-*trans*-β-carotene and *neo*-β-carotene U, while extracts from red and yellow vegetables contained all-*trans*-β-carotene and *neo*-β-carotene B [63]. In 1971, these authors reported that thermal processing of vegetables increased the relative amounts of *cis* isomers detected [64].

The method of Sweeney and Marsh [63] was used by others to investigate the effects of processing. Lee and Ammerman [65] evaluated the effects of several canning processes on the β-carotene composition of sweet potatoes. The relative amount of *neo*-β-carotene B was found to increase significantly with canning. Contrary to results with other plant tissues, Gebhart *et al.* [66] found heat processing of peaches did not effect isomer composition. Ogunlesi and Lee [67] reported increases in *cis* isomer content, with concurrent loss of all-*trans*-β-carotene, when carrots were processed. More recently, Van der Pol *et al.* [68] reported increased relative isomer content after traditionally cooking Indonesian vegetables.

Vecchi *et al.* [69] developed an HPLC method based on adsorption chromatography, using an alumina (Al_2O_3) column with an eluent of hexane. The system was designed such that the hexane eluent had a controlled water content. Using NMR to identify geometrical configurations, they found 9-*cis*-β-carotene, 13-*cis*-β-carotene and 15-*cis*-β-carotene to be the main *cis* isomers in isomerized β-carotene preparations. Five minor isomers (mainly di-*cis*) were also isolated and identified. The 9-*cis* and 13-*cis* isomers were the main *cis* isomers in carrots and paprika, with none of the other isomers being detected at levels higher than 2% of the total carotene.

Tsukida *et al.* [70], using NMR, confirmed that the configurations of the *neo*-β-carotene B and *neo* β-carotene U from $Ca(OH)_2$ open column chroma-

tography were 13-*cis*- and 9-*cis*-β-carotene, respectively. Based on this open column method, Tsukida *et al.* [71] developed an HPLC method employing a $Ca(OH)_2$ column with a mobile phase of hexane containing 0.1–2.0% acetone. The authors identified nine *cis* isomers of β-carotene in thermally isomerized β-carotene. With this method, the sterically hindered 7-*cis*-β-carotene, which was thought to be too labile to isolate, was identified [72]. Later, Ca $(OH)_2$ columns of greater resolution were produced [73] and these allowed for the further identification and reassignment of certain di- and tri-*cis* isomers, using ^1H NMR. Fig. 3 illustrates this separation.

Chandler and Schwartz [74] using a $Ca(OH)_2$ HPLC column method similar to Tsukida *et al.* [71] reported on the isomeric composition of carotenoid extracts from fresh and processed fruits and vegetables. The main *cis* isomers of β-carotene detected were 9-*cis* and 13-*cis*. Chandler and Schwartz [75] used this method to evaluate the effects of various processing techniques on the β-carotene isomer composition of sweet potato. Severe heat treatments resulted in higher relative percentages of isomers (dehydration, 28.9%; and baking, 23.0%) as compared to milder processing techniques (canning, 17.2%; microwaving, 16.5%; and blanching

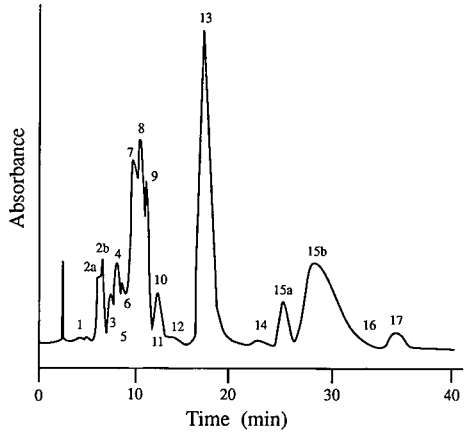

Fig. 3. Chromatographic separation of thermally isomerized β-carotene using $Ca(OH)_2$ packed column and mobile phase of 0.1% acetone in *n*-hexane. Peaks: 2 = 13,15-*cis*-β-carotene, 3 = 15-*cis*-β-carotene, 4 = 13,13′-*cis*-β-carotene, 7 = 13-*cis*-β-carotene, 8 = 9,15-*cis*-β-carotene, 9 = 9,13′-*cis*-β-carotene, 10 = 9,9′,13-*cis*-β-carotene, 12 = 7,13′-*cis*-β-carotene, 13 = all-*trans*-β-carotene, 14 = 7,9-*cis*-β-carotene, 15a = 9,9′-*cis*-β-carotene, 15b = 9-*cis*-β-carotene and 17 = 7-*cis*-β-carotene (the other peaks were not identified). From Koyama *et al.* [73].

10 min, 15.3%). As was seen by Lee and Ammerman [65], 13-*cis*-β-carotene (*neo*-β-carotene B) was the predominant isomer formed. Pettersson and Jonsson [76], using similar methods, reported fresh carrot juice did not contain significant *cis* isomers, while heat processing the juice resulted in the detection of several *cis* isomers. Thus, as was seen by earlier researchers employing the method of Sweeney and Marsh [63], these researchers using HPLC methods found that heat processing increases *cis* isomer content.

Most of the reported normal-phase separations of β-carotene isomers involved $Ca(OH)_2$ columns. The mobile phase usually contained small amounts of acetone in hexane, however, Craft *et al.* [77] preferred to use pentane as the main solvent. Sometimes a gradient of increasing acetone content is necessary to achieve the desired selectivity of earlier peaks yet elute the more retained isomers (*i.e.* 9-*cis*-β-carotene). For example, Tsukida *et al.* [71] started their elution with 0.1% acetone in hexane and finished with 2.0% acetone in hexane. $Ca(OH)_2$ columns are not commercially available. Therefore, slight differences in packing techniques and handling procedures for the $Ca(OH)_2$ result in column to column variability [78]. Thus, adjustments in mobile phase composition is often required to obtain optimum resolution for a particular column.

One concern with normal-phase methods is that the water activity of the stationary phase is thought to be important in the resolution of the isomers. This was first investigated by Bickoff *et al.* [21] for open $Ca(OH)_2$ column chromatography. It was reported that for optimum separation, a relative humidity in the range of 30–50% is desirable. Schwartz and Patroni-Killam [62] found that for TLC plates equilibration to a relative humidity (RH) of 44% was required to obtain optimum resolution. Chandler and Schwartz [74] also used Ca $(OH)_2$ equilibrated to a RH of 44% to pack HPLC columns. An increase in the separation of some minor *cis* isomers was found by O'Neil *et al.* [78] when the $Ca(OH)_2$ was hydrated first and then equilibrated to 44% RH. Another approach to control water activity was taken by Petterson and Jonsson [76]. $Ca(OH)_2$ was dried at 105°C and stored in a desiccator prior to packing HPLC columns. While there is a need to control water activity for Ca $(OH)_2$ column separations, the precautions re-

quired are not as elaborate as those employed for alumina column chromatography in a moisture-controlled isolated HPLC system [69].

The mobile phase or injection solvent can change the water activity of the stationary phase, and water needs to be removed, especially from sample extracts. This can be done by adding anhydrous sodium sulfate followed by filtration to remove the crystals and bound water [79]. Pettersson and Jonsson [76] reconditioned $Ca(OH)_2$ columns that may have adsorbed water by evaporating solvent with a stream of nitrogen. drying in an oven at 105°C, and flushing the column with a stream of pure nitrogen. On the other hand, it has been observed in our laboratory that when some $Ca(OH)_2$ columns lose the ability to resolve the *cis* isomers, adding water to the mobile phase (0.05%) often returns activity. Thus, it appears the loss of water from the surface of the stationary phase can also effect the separation.

3.3. Development of modern HPLC methods and their use in plant tissue analyses: reversed-phase

Several reversed-phase methods have been reported that separate geometrical isomers of β-carotene. Most of these methods involve the use of Vydac C_{18} TP columns (either a Vydac 201TP or a Vydac 218TP column). These columns are produced using trichlorosilanes, which is referred to as a polymeric synthesis of C_{18} reversed-phase columns. When monochlorosilanes are used, the column is said to be a monomeric column. These Vydac columns have a silica base that is spheroidal with a pore size of 300 Å and a surface area of 80 m^2/g. The wide bore is recommended for the separation of large bio-molecules. The low surface area results in a medium capacity reversed-phase column for the separation of very hydrophobic small molecules. The packing material of the Vydac 218TP column is endcapped with trimethylsilane after the C_{18} chain is bonded to the TP silica to prevent adsorption of polar compounds. No endcapping is performed for the Vydac 201TP column and thus is recommended for very nonpolar compounds.

Jensen *et al.* [80] resolved several β-carotene isomers employing a Nucleosil C_{18} column and a mobile phase of acetone–water (88:12). Analyzing solutions of all-*trans*-β-carotene photoisomerized in the presence of chlorophyll *a*, revealed the formation of three isomers, which were identified as 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene based on their absorption spectra and coelution with an authentic standard of 15-*cis*-β-carotene. The main isomers formed were 9-*cis*- and 13-*cis*-β-carotene, with the 9-*cis* isomer generally being the most predominant *cis* isomer. This method of separation was attempted in our laboratory, however, the 13-*cis* and 15-*cis* isomers coeluted and the 9-*cis* isomer eluted with all-*trans*-β-carotene [78].

Bushway [81] compared various reversed and normal-phase HPLC methods and concluded that Vydac C_{18} columns provided quicker and better separation of carotenoids. Several methods were found that separated at least three β-carotene peaks. Bushway [82], using the Vydac 218TP column and an eluent of methanol–acetonitrile–tetrahydrofuran (56:40:4), analyzed several carotenoid extracts from raw fruits and vegetables. Iodine isomerized β-carotene was analyzed and spectra of chromatographic peaks were used for peak identification. Both 15-*cis*- and 9-*cis*-β-carotene were detected.

Pesek and co-workers [83,84] employed similar methods to investigate the isomerization of β-carotene in organic solvents and during illumination of solutions. The mobile phase was slightly different being methanol–acetonitrile–tetrahydrofuran (58:42:1). They first investigated the effect of sample solvent on spontaneous isomerization [83]. All-*trans*-β-carotene was stored for 24 h at 25°C in the dark. Their results confirmed previous concerns that traces of HCl in chloroform may result in isomerization [9]. Both methylene chloride and chloroform resulted in a 20–30% loss in all-*trans*-β-carotene. On the other hand, the mobile phase solvents could be used without extensive isomerization ($\leq$5% loss of all-*trans*-β-carotene). The kinetics of isomerization in the mobile phase at 45°C (in the dark) was also investigated [83]. After 4–6 days, an equilibrium mixture of isomers was reached, with all-*trans*-β-carotene constituting 67% of the β-carotene. The two *cis* isomers formed during isomerization were 9-*cis*-carotene and 13-*cis*-β-carotene. The rate of formation and equilibrium concentration of 13-*cis*-β-carotene was higher than that of 9-*cis*-β-carotene. Contrary to these results, when β-carotene was exposed to light (at 28°C), the photoisomerization resulted in the formation of more

9-*cis*-β-carotene than 13-*cis*-β-carotene [84]. However, it was noted that the rate of photodegradation under these conditions was much greater than the rate of photoisomerization.

Quackenbush and Smallidge [85] using the Vydac 201TP C_{18} column with a mobile phase of methanol–chloroform (90:10) resolved the *cis* isomers of β-carotene from the all-*trans* isomer. Quackenbush [86] investigated the β-carotene content of several fruits and vegetables, using a Vydac 201TP column and a gradient of methanol for 5 min followed by methanol–chloroform (94:6) or for isocratic analysis, methanol–chloroform (94:6). Fig. 4 is a chromatogram of an extract from a canned carrot sample, separated using the isocratic mobile phase. Spectral data and results from an analysis of iodine isomerized β-carotene were used to identify resolved β-carotene peaks. It was found that the 9-*cis*, 13-*cis*, and all-*trans* isomers were resolved. Results from analyses of plant extracts were similar to Sweeney and Marsh [63] with 9-*cis*-β-carotene being detected at relatively higher amounts in extracts from green vegetables and more 13-*cis*-β-carotene found in extracts from yellow vegetables. This author also found a larger percentage of *cis* isomers in processed products, in agreement with Chandler and Schwartz [74] and Sweeney and Marsh [64].

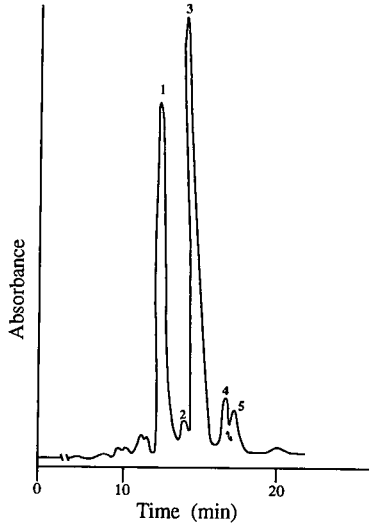

Fig. 4. Chromatographic separation of an extract from canned carrots using a Vydac 201TP C_{18} column and a mobile phase of methanol–chloroform (94:6). Peaks: 1 = all-*trans*-α-carotene, 2 = *cis*-α-carotene, 3 = all-*trans*-β-carotene, 4 = 13-*cis*-β-carotene and 5 = 9-*cis*-β-carotene. From Quackenbush [86].

Saleh and Tan [87] worked on optimizing a mobile phase to separate a number of carotenoid *cis/trans* isomers. A mobile phase of acetonitrile–methanol–methylene chloride (80:18:2) led to separation of several *cis* isomers of nonpolar carotenoids using a Zorbax column and also separated β-carotene isomers when a Vydac 218TP column was used. *Cis* isomers of β-carotene (identified as 9-*cis* and 13-*cis*-β-carotene) were detected from analyses of palm and carrot oils.

Another optimization of carotene *cis/trans* isomer separations was reported by Lesellier *et al.* [88]. An eluent of acetonitrile–methanol–methylene chloride (63:27:10) was used with a polymeric C_{18} column. As was found by others [77,81,85], the polymeric C_{18} column (Brownlee ODS RO 18) provided greater separation of the *cis/trans* isomers. It is interesting to note that the *cis* isomers eluted prior to the all-*trans* isomer on a monomeric Brownlee OD RP 18 column. This is in contrast to reports by others using monomeric columns, where the appearance of a *cis* isomer shoulder following the β-carotene peak was seen [45,89].

Polymeric C_{18} columns appear to offer the best reversed-phase separation of β-carotene *cis/trans* isomers, with Vydac C_{18} columns being the most often reported. It has been speculated that the wide bore of the polymeric columns allows greater interaction of the 33 Å long β-carotene molecule with the bonded phase [77,90]. Of the Vydac polymeric columns, the non-endcapped Vydac 201TP appears to offer greater resolution [78,85]. However, several researchers have had success in resolving the main *cis* isomers with endcapped columns [81,83,84,87]. An advantage of endcapped Vydac columns is short elution times [81].

One disadvantage of using polymeric C_{18} columns is that column to column variability tends to occur to a greater extent than for monomeric columns [91]. Resolution among the isomers with Vydac C_{18} columns has been reported to vary [78]. Another factor that can limit full resolution of the isomers on non-endcapped Vydac C_{18} columns is deposition of lipid material on the column. It appears that the insolubility of nonpolar compounds in a mobile phase of primarily methanol results in lipid material remaining on the column. This results

in decreased interaction of the carotenes with the stationary phase and loss of resolution [78]. In our laboratory, washing the columns frequently with methanol–chloroform (50:50) maintains or restores the original separation.

For the predominant methods employing normal-phase separations, NMR investigations were performed to identify the carotene isomers [69,73]. This is not the case for most reports of reversed-phase separations. The fact that positive identification is not always performed may result in the misidentification of certain isomers. The presence of a shoulder eluting after all-*trans*-β-carotene has been reported by a number of authors using monomeric C_{18} reversed-phase HPLC [45,46,89,92–94]. Speculations on the identity of this shoulder have been made based on coelution with standards or absorption spectra. However, this shoulder may contain a combination of isomers. For several monomeric C_{18} reversed-phase separations, standards of the main *cis* isomers (13-*cis*-, 15-*cis*-, and 9-*cis*-β-carotene) all eluted in the shoulder, demonstrating the lack of separation by these columns [78]. In addition to coelution problems with monomeric columns, several of the polymeric C_{18} reversed-phase methods have been reported to coelute 13-*cis*- and 15-*cis*-β-carotene [78].

The *cis* isomer eluting as a shoulder immediately following all-*trans*-β-carotene is observed when employing monomeric columns and has been identified as 15-*cis*-β-carotene in several fruit and vegetable samples [43,45,94,95]. These identifications were based on coelution with an authentic 15-*cis*-β-carotene standard. This standard has been readily available, because it is the precursor to all-*trans*-β-carotene in the organic synthesis of β-carotene [96]. Due to the accessibility of this *cis* isomer, while commercial standards of the other *cis* isomers were not attainable, the retention time of only this isomer could be confirmed. However, other researchers employing polymeric columns or $Ca(OH)_2$ columns have found that the 13-*cis* and 9-*cis* isomers are more prevalent in plant tissues [74,86]. Thus, the reports of the 15-*cis* isomer may in actuality include other isomers.

One advantage of reversed-phase chromatography is that additional handling to ensure complete removal of water from the sample is not required. However, in the case of the analysis of carotene, additional sample handling is often required. This is necessary because problems can arise when the sample solvent is not miscible with the mobile phase [97]. Nonpolar solvents [polarity (P') < 5] are often used for the extraction of carotenes. These solvents may not be miscible with relatively polar mobile phases (P' > 5), and this may lead to artifact formation [97]. From investigations in this laboratory, the method found to prevent this problem is to evaporate the nonpolar solvent, redissolve in ethyl ether and dilute with methanol to a proportion of 20:80 (P' = 4.6). This sequence of solvent transfers results in a carotene solution which is miscible with the mobile phase. This procedure adds extra handling, however, improved recovery and chromatographic separation is achieved.

While some of the reported reversed-phase separations utilize large amounts of acetonitrile in the mobile phase, it appears that using methanol as the primary solvent for carotenoid analysis results in greater selectivity [77]. In addition, low recoveries have been reported when acetonitrile was employed as the primary solvent. Craft *et al.* [77] reported only 74% recovery of β-carotene with an acetonitrile-based solvent; however, recoveries increased to 89% with a methanol-based mobile phase. Even though similar solubilities for β-carotene in methanol and acetonitrile were measured [98], between the two, methanol appears to be the solvent of choice to maximize sample recovery. On the other hand, the addition of small amounts of ammonium acetate (0.01%) to the mobile phase has been found to increase recoveries, when an acetonitrile-based mobile phase is used for carotenoids [99].

Temperature for optimum selectivity values was reported to be between 20 and 25°C for a polymeric C_{18} column with a mobile phase of acetonitrile–methanol–methylene chloride (45:45:10) [88]. It was found that the selectivity (α) for *cis*-α-carotene/α-carotene was inversely related to temperature, while α for β-carotene/α-carotene and *cis*-β-carotene/β-carotene decreased as temperature decreased. A column temperature of 20°C was also selected by Craft *et al.* [100] as the optimum for separating a mixture of carotenoids with a polymeric C_{18} column and a mobile phase of methanol–tetrahydrofuran (95:5).

3.4. Analyses of serum, plasma and human tissues

Bieri *et al.* [54] developed an HPLC method to detect additional carotenoids that had not been previously reported in plasma. The separation employed a C_{18} column with an eluent of acetonitrile–methylene chloride–methanol (70:20:10). A photodiode array detector was used in the method development and it was noted by these authors that the tail of the β-carotene peak contained 15-*cis*-β-carotene. This identification was based on its high absorbance at 340 nm. Sowell *et al.* [101], using a Microsorb C_{18} column and a mobile phase of ethanol–acetonitrile (1:1), detected a *cis* isomer of β-carotene in human serum. The β-carotene *cis* isomer was suggested to be 15-*cis*-β-carotene based on the previous report by Bieri *et al.* [54].

Jensen *et al.* [102] investigated the effects of ingesting *cis* isomers of β-carotene on serum levels in humans. A *cis* isomer peak was resolved from all-*trans*-β-carotene with a Vydac 201TP C_{18} column and an eluent of methanol–acetonitrile (90:10). The amount of all-*trans*-β-carotene was compared to *cis*-β-carotene in the serum of humans fed a β-carotene preparation from *Dunaliella* algae, a source of β-carotene that is high in the 9-*cis* isomer [103]. There was not a large increase in *cis*-β-carotene in the serum of these individuals.

Rushin *et al.* [104], in their analysis of human sera, also resolved *cis* isomers of β-carotene from the all-*trans* form. Two Vydac 201TP C_{18} columns connected in series were used with a mobile phase of methanol–chloroform–tetrahydrofuran (87:10:3). Standards of all-*trans*, 9-*cis* and 13-*cis*-β-carotene were isolated using a semi-preparative $Ca(OH)_2$ column separation and their identities confirmed using absorption spectra and ^{1}H-NMR. For serum samples, a collective *cis* isomer peak was separated from the all-*trans*-β-carotene, but individual *cis* isomers were not resolved. The standards of 13-*cis*- and 9-*cis*-β-carotene coeluted with this *cis* peak. Absorption spectra from serum samples suggested that 13-*cis*-β-carotene is the predominant *cis* isomer in human sera, however small quantities of other isomers may have been present in the serum extracts. The relative amount of *cis* isomers compared to all-*trans*-β-carotene in the serum is similar to that found for a mean value calculated from a variety of fruits and vegetables. These results indicate that the carotenes are likely absorbed as the geometrical configuration in which they were ingested.

Another method to resolve the *cis* isomers from all-*trans*-β-carotene was reported by MacCrehan and Schönberger [105]. These authors developed a multivitamin serum assay to quantitate retinol, α-tocopherol and β-carotene. A Vydac 201TP C_{18} column was used with a gradient of methanol–water–*n*-butanol (75:15:10) for 3 min followed by a linear increase to methanol–water–*n*-butanol (88:2:10) over 15 min. In contrast to Rushin *et al.* [104], a later article [90] identified the *cis* isomer peak as 9-*cis*-β-carotene.

Stahl *et al.* [106] were able to separate 13-*cis*- and 15-*cis*-β-carotene as one peak from all-*trans*-β-carotene for serum samples. These authors used a C_{18} endcapped column (E. Merck, Germany) with a mobile phase of methanol–acetonitrile–methylene chloride–water (7:7:2:0.16). This separation is illustrated in Fig. 5. Another isomer, 9-*cis*-β-carotene, was observed in many of the human tissues analyzed (liver, kidney, adrenal glands, and testes), however, it was not found in the serum samples. Since 13-*cis*-β-carotene and 15-*cis*-β-carotene were not resolved under these conditions, it is not clear whether 15-*cis*-β-carotene is a prevalent isomer in human plasma or tissues. Previous reports of 15-*cis*-β-carotene in serum [54,101] employed monomeric

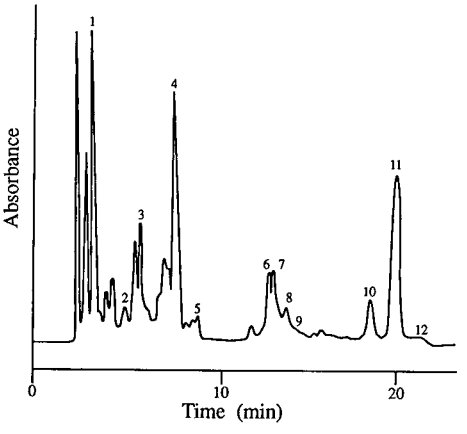

Fig. 5. Chromatographic separation of an extract from human serum using a C_{18} column with a mobile phase of methanol–acetonitrile–dichloromethane–water (7:7:2:0.16). Peaks: 1 = zeaxanthin, 2 = canthaxanthin, 3 = standard, 4 = cryptoxanthin, 5 = *cis*-cryptoxanthin, 6 = lycopene, 7 = 9-*cis*-lycopene, 8 = 13-*cis*-lycopene, 9 = 15-*cis*-lycopene, 10 = α-carotene, 11 = all-*trans*-β-carotene and 12 = 13-,15-*cis*-β-carotene. From Stahl *et al.* [106].

C_{18} column methods which have been shown to coelute the *cis* isomers of β-carotene [78].

3.5. Algal sources of carotenes

Ben-Amotz *et al.* [103] used an HPLC alumina column (Alox-T) with an eluent of hexane–methylene chloride (65:35) to analyze the algae species *Dunaliella salina* and *Dunaliella bardawil*. The authors reported that the algae accumulated approximately as much 9-*cis*-β-carotene as all-*trans*-β-carotene. Smaller quantities of other isomers were detected, with one isomer identified as 15-*cis*-β-carotene. Later, similar results were found using the method of Jensen *et al.* [102] employing a Vydac 201TP C_{18} column and a mobile phase of methanol–acetonitrile (90:10) [107]. A typical chromatogram illustrating the separation of the algal carotenoids is shown in Fig. 6. Watanabe and Hayashi [108] using a YMC pack ODS-A column and a mobile phase of acetonitrile–methanol–*n*-hexane–ethyl ether (70:15:10:5) also detected high amounts of 9-*cis*-β-carotene present in *Dunaliella* powder.

Ben-Amotz *et al.* [109,110] have been investigating the use of β-carotene from *Dunaliella* algae as a source of natural β-carotene. Rats and chicks fed β-carotene from *Dunaliella bardawil* satisfied the total requirements for retinol. Liver assays showed at least a ten-fold increase in β-carotene storage for both species when they were fed the algae source of carotene as compared to animals fed a diet containing synthetic all-*trans*-β-carotene [111]. The ratio of 9-*cis*- to all-*trans*-β-carotene in the liver was approximately the same as the algae β-carotene preparation. To determine if this greater storage level

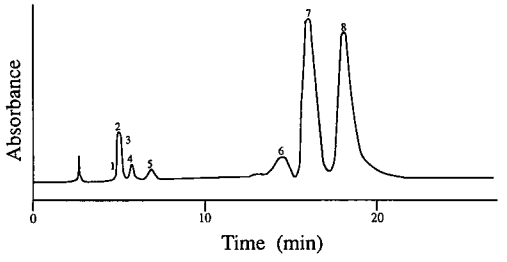

Fig. 6. Chromatographic separation of an extract from *Dunaliella bardawil* algae using a Vydac 201TP C_{18} column and a mobile phase of methanol–acetonitrile (90:10). Peaks: 1 = chlorophyll *b*, 2 = lutein, 3 = unidentified, 4 = zeaxanthin, 5 = chlorophyll *a*, 6 = α-carotene, 7 = all-*trans*-β-carotene and 8 = 9-*cis*-β-carotene. From Ben-Amotz *et al.* [110].

was an effect of the 9-*cis* isomer or the result of other components present in the algae, partially purified 9-*cis*-β-carotene was collected and fed to chicks [112]. Chicks fed 9-*cis*-β-carotene stored ten times more β-carotene in the liver than those fed synthetic all-*trans*-β-carotene. Greater storage of β-carotene was found to have no adverse effect on retinol levels in the liver. The authors speculated that the 9-*cis* isomer is more fat-soluble and more difficult to crystallize and thus, may increase the absorption and deposition of the β-carotene.

This work by Ben-Amotz and co-workers [111,112] indicates that 9-*cis*-β-carotene may be absorbed and deposited in the tissues. The results of Rushin *et al.* [104] also suggest that *cis* isomers are likely absorbed along with all-*trans*-β-carotene and circulate in the blood. However in humans, Jensen *et al.* [102] found little increase in the relative amount of *cis*-β-carotene in the serum of adults fed algae extracts high in *cis*-β-carotene.

4. ANALYSES OF OTHER CAROTENOID *CIS/TRANS* ISOMERS

4.1. Early HPLC methods

The number of reports concerning *cis* isomers of carotenoids besides β-carotene has not been as extensive. However, chromatographic separation of other isomers is beginning to be addressed and should lead to more reports regarding other carotenoids. One of the few earlier reports detecting *cis* isomers was by Matus *et al.*, in 1981 [113]. These authors used a multiple wavelength UV–visible detector to detect *cis* peak absorbances. *Cis* isomers of violaxanthin, lutein and capsanthin were separated from their all-*trans* forms using a 10-μm C_{18} Nucleosil column and a gradient of acetone–water (100:40 then 100:5). The source from which these *cis* isomers were extracted was a Canadian waterweed, *Elodea canadensis*.

Ruddat and Will [114], evaluating HPLC techniques for the analysis of carotenoids, detected *cis*-β-zeacarotene in an extract from a yellow strain of sporidia, and *cis*-lycopene and *cis*-ζ-carotene from a tomato extract. A gradient of 2-propanol and acetonitrile–water (9:1) from a ratio of 30:70 to 55:45 with a C_{18} Ultrasphere column was used for separation. Fractions were collected and absorption spectra were recorded to aid in identification of chromatographic peaks.

4.2. Analyses of canthaxanthin cis/trans isomers

Nelis *et al.* [115] detected relatively large quantities of *cis*-canthaxanthin in the encysted embryos of the brine shrimp *Artemia*. It was found that *cis*-canthaxanthin was found in only the ovaries, eggs and the hemolymph of reproductively active females. It was speculated that these isomers may play a specific role in reproduction or embryo development.

Because of these findings, Nelis *et al.* [116] investigated the possibilities of improving both normal-phase and reversed-phase separation of these isomers. The optimum reversed-phase separation occurred with a Zorbax C_{18} column and a mobile phase of methanol–acetonitrile–methylene chloride (50:41:9). However, the *cis* isomers eluted as one peak. Normal-phase separation with a silica column and a mobile phase of methylene chloride–2-propanol (99.3:0.7) enabled the detection of 13-*cis*-, 15-*cis*- and 9-*cis*-canthaxanthin. While this normal-phase method provided the best separation for qualitative investigations, the authors noted that extra handling required to transfer extracts to hexane for HPLC analyses and the contact with acidic silica resulted in a quantitative loss of *cis* isomers. Thus, it was recommended that reversed-phase be used for quantitative analysis and normal phase for qualitative investigations.

Hashimoto and Koyama [117] also separated canthaxanthin isomers by normal-phase chromatography. $Ca(OH)_2$ columns similar to those prepared for β-carotene separations [73] were employed. The mobile phase was benzene–hexane (95:5) instead of acetone in *n*-hexane, as is used for β-carotene separations. Using ^1H NMR, they identified three mono-*cis* isomers (13-*cis*-, 15-*cis*-, and 9-*cis*-canthaxanthin) and four di-*cis* isomers, isolated from heated canthaxanthin crystals.

Another reversed-phase separation was reported by Mayne and Parker [118]. They used a C_{18} Ultrasphere column with mobile phase of ethanol–water (95:5). One peak was identified as *cis*-canthaxanthin with other peaks as possible *cis* isomers of canthaxanthin. These three peaks were observed in analyses of canthaxanthin beadlet preparations. When these beadlets were fed to chicks, the same compounds were found in liver and membrane extracts, indicating that *cis* isomers are absorbed and deposited intact.

4.3. Development of HPLC methods and their use in plant tissue analyses

Chandler and Schwartz [74] detected *cis* isomers of α-carotene in canned carrots, however, because only small quantities were found and complete resolution was not achieved, these isomers were not unequivocally identified. Pettersson and Jonsson [76] also found α-carotene isomers to be present in heat processed carrot juice when using a $Ca(OH)_2$ packed column. Photodiode array detection was used to tentatively identify two of these isomers as 13-*cis*-α-carotene and 9-*cis*-α-carotene.

Quackenbush [86] performed iodine-catalyzed isomerization of α-carotene, lutein and lycopene. The resulting isomeric mixtures were separated using a Vydac 201TP column and a mobile phase of methanol and/or methanol–chloroform (94:6). Similar to β-carotene, the predominant isomers of α-carotene were 13-*cis*- and 9-*cis*-α-carotene. An additional isomer was found for lutein, which was identified as 13′-*cis*-lutein. Five *cis* isomers of lycopene were detected, however, due to insufficient data in the literature, there was no attempt to identify the configuration of these isomers.

Results from our laboratory have shown that with similar HPLC methods other carotenoid isomers are separated (unpublished data). Fig. 7 illustrates the separation of at least three *cis*-γ-carotene

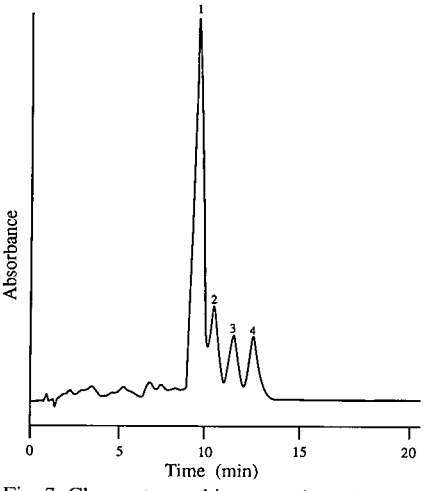

Fig. 7. Chromatographic separation of an extract from Indonesian oncom using a Vydac 201TP C_{18} column and a mobile phase of methanol. Peaks: 1 = γ-carotene and 2–4 = *cis*-γ-carotene.

isomers and all-*trans*-γ-carotene with an eluent of methanol and a Vydac 201TP C_{18} column. These isomers were found in oncom, which is an Indonesian food product of peanut presscake fermented by *Neurospora sitophila*. Additionally, several neurosporene and β-cryptoxanthin isomers have been separated with this procedure.

Saleh and Tan [87] developed a mobile phase that separated a number of *cis*/*trans* carotenoid isomers. For separating β-carotene isomers, the polymeric Vydac 218TP C_{18} column was most effective, however, for the more polar carotenoids, a Zorbax C_{18} column provided better resolution. The optimum solvent composition selected was acetonitrile–methanol–methylene chloride (80:18:2). Two isomers of lutein (13-*cis* and 9-*cis*) were found in carrot oil samples, along with *cis* isomers of γ-carotene, ζ-carotene, phytofluene, and phytoene. The analysis of palm oil carotenoids revealed *cis*-γ-carotene, *cis*-ζ-carotene and *cis*-phytoene, as well as four *cis* isomers of lycopene.

Khachik *et al.* [119] detected *cis*-phytoene and *cis*-phytofluene in dried apricots and peaches. These were detected with both a gradient elution monomeric C_{18} column (Microsorb) separation and an isocratic Vydac C_{18} column system. Both solvent systems included acetonitrile–methanol–methylene chloride–hexane (85:10:2.5:2.5). After 10 min of this mobile phase, the gradient separation linearly increased, over 30 min, to a composition of acetonitrile–methanol–methylene chloride–hexane (45:10:22.5:22.5). *Cis*-β-carotene and *neo*-γ-carotene were also detected in dried apricots, when the Vydac column system was employed. Using this isocratic separation, *neo*-lycopene was found in extracts of pink grapefruit. Employing the described gradient system, two *cis* isomers of lutein, *cis*-neoxanthin and *cis*-β-carotene were found in extracts of several raw and cooked green vegetables [48]. Also, *cis* isomers of phytoene, phytofluene and β-carotene were detected in extracts of tomato paste.

4.4. Analyses of serum, plasma and animal tissues

Sowell *et al.* [101] were able to obtain baseline separation between a *cis* isomer of β-carotene and all-*trans*-β-carotene, and two *cis* isomers of zeaxanthin and all-*trans*-zeaxanthin for extracts of human serum. A Microsorb C_{18} column with an eluent of ethanol–acetonitrile (1:1) was used for this separation. Shoulders following the all-*trans* form were identified as *cis* isomers of cryptoxanthin, lycopene and α-carotene.

Krinsky *et al.* [120] employed an Alltech Adsorbosphere HS C_{18} column and a gradient of solvent A [acetonitrile–methanol with 0.01% ammonium acetate (85:15)] for 10 min followed by solvent A–isopropanol (70:30) to separate carotenoids from human plasma. Several *cis* isomers of lycopene were partially separated, but these isomers were not identified. For both lutein and zeaxanthin, the 13-*cis* isomer was identified. It was noted that a peak appearing to be 9-*cis*-zeaxanthin was detected when an extract of corn meal was analyzed. However, this peak was too small to quantitate for the serum sample. Similar results were found for analyses of monkey plasma [121]. The authors noted that the 9-*cis* isomer of zeaxanthin was present in the diet, but not detected in plasma extracts.

Stahl et al. [106], in addition to reporting the detection of β-carotene isomers, found *cis*-cryptoxanthin and 9-*cis*-, 13-*cis*- and 15-*cis*-lycopene in serum samples and in several tissues from humans. These were identified using visible absorption spectra. The separation was a reversed-phase C_{18} separation with a mobile phase of methanol–acetonitrile–methylene chloride–water (7:7:2:0.16). Schmitz *et al.* [122], using a Vydac C_{18} column and a mobile phase of methanol–acetonitrile–water (88:9:3), also found *cis*-lycopene in human tissues (kidney, liver, and lung tissues). However, individual isomers were not separated. While Stahl *et al.* [106] did not find large amounts of 9-*cis*-β-carotene in serum, 9-*cis*-lycopene was found at nearly the level of the all-*trans* form. The ratio of 9-*cis*/all-*trans* for both carotenoids varied among the tissues.

Using two different HPLC systems, Khachik *et al.* [51] separated seventeen carotenoids from human plasma. Polar carotenoids were separated on a silica-based nitrile-bonded column with a mobile phase of hexane–dichloromethane–methanol–N,N-diisopropylenethylamine (74.65:25.00:0.25:0.10). The reversed-phase C_{18} separation employed a gradient of acetonitrile–methanol–dichloromethane–hexane (85:10:2.5:2.5) for 10 min followed by a linear gradient over 30 min to acetonitrile–dichloromethane–hexane–methanol (45:22.5:22.5:10). *cis*-3-Hydroxy-β,ε-carotene-3′-one, 9-*cis*-lutein, 9′-*cis*-lutein, 13-*cis*- and/or 13′-*cis*-lutein, and 9-*cis*-, 13-*cis*-

and 15-*cis*-zeaxanthin were reported to be detected in human plasma using the nitrile-bonded column. *cis*-5,6-Dihydroxy-5,6-dihydrolycopene, *cis*-2′,3′-anhydrolutein, *cis*-lycopene, *cis*-neurosporene, *cis*-β-carotene and *cis*-phytofluene were found when the reversed-phase system was employed. The identification of these carotenoids was based on UV–visible spectra and by comparison of retention times to thermally isomerized synthetic carotenoids. As was found by Krinsky *et al.* [120], the ratio of *cis* isomers to the all-*trans* isomer was smaller for lutein than for zeaxanthin and slightly more 13-*cis*-zeaxanthin than 9-*cis*-zeaxanthin was detected.

4.5. Supercritical fluid chromatography

Separation of the geometrical isomers of β-carotene and α-carotene with supercritical fluid chromatography (SFC) has been shown by Schmitz *et al.* [123]. Several β-carotene isomers were separated with a SB-cyanopropyl-25-column (25% cyanopropyl and 75% polymethylsiloxane cross-linked stationary phase). To resolve isomers of α-carotene, the authors used two SB-cyanopropyl-50-column (50% cyanopropyl and 50% polymethylsiloxane cross-linked stationary phase). For both of these separations, the mobile phase was supercritical carbon dioxide with 1% ethanol. Positive identification of the isomers has not yet been reported; however, the degree of separation achieved by these authors demonstrates the potential for the use of SFC to separate geometrical isomers.

Other researchers have been examining SFC for use in carotene separations, including an optimization varying temperature, pressure, and organic modifiers in the supercritical fluid [124]. An α-carotene *cis* isomer was resolved from all-*trans*-α-carotene as well as two *cis* isomers of β-carotene were separated from all-*trans*-β-carotene with a reversed-phase column. The analysis time was shorter than that achieved by the HPLC reversed-phase method previously reported by this group of researchers [88]. The density of carbon dioxide changed the efficiency of the separation but did not affect the selectivity. On the other hand, modifiers in the supercritical fluid affected the selectivity and not the efficiency. These authors also investigated the effects of stationary phase on the selectivity and retention of carotene when using carbon dioxide with methanol as a modifier [125]. Polymeric C_{18} columns were able to resolve the *cis* isomers of α and β-carotene from the all-*trans* isomers, but this was not achieved with monomeric columns. This conclusion is similar to results found with HPLC methods [77,81,85].

5. CONCLUSIONS

5.1. Separation of carotenoid isomers

For the separation of β-carotene isomers, most procedures employ either a $Ca(OH)_2$ or a Vydac C_{18} column. These two types of columns have been found to offer better separation of these isomers compared to other columns [77,78]. A comparison of HPLC methods for the analysis of plant tissues was reported by O'Neil *et al.* [78]. The reversed-phase method, selected as offering the best separation, employed a Vydac 201TP C_{18} column and the gradient elution conditions reported by Quackenbush [86]. The normal-phase $Ca(OH)_2$ column method was found to be less sensitive for quantitative measurements than the selected reversed-phase method, but offered greater selectivity for the carotenes. Thus, the procedure employing a $Ca(OH)_2$ column was selected as the preferred method for the analysis of β-carotene *cis*/*trans* isomers in plant tissues. Unfortunately, this method was found to be inappropriate for the analysis of plasma β-carotene isomers [104]. Of the HPLC methods discussed for the separation of β-carotene *cis* isomers in human plasma or tissues, the method of Stahl *et al.* [106] appears to offer the best resolution.

Chromatographic methods enabling the detection of *cis*-carotenoids usually involve reversed-phase separations. In general, polymeric C_{18} columns allow for the detection of *cis*-carotenes [78,86,87], while monomeric columns provide for some separation of certain xanthophylls [87,119]. However, for more complete separation of specific carotenoids, several normal-phase separations may be more appropriate. For example, $Ca(OH)_2$ columns could be used for α-carotene [76] and canthaxanthin separations [117]. Recently, an HPLC method employing silica-based nitrile columns [48] has shown potential for the separation of polar carotenoids and SFC methods [123–125] have resolved carotene isomers.

5.2. Limitations of HPLC methods

This review has presented a number of chromato-

graphic methods which enable the detection of carotenoid *cis* isomers. However, many of these separations do not afford baseline resolution of the *cis/trans* isomers being reported and identification of the *cis* forms may be tentative and unconfirmed. In addition, coelution of isomers may prevent the detection of other geometrical forms. An example of this situation, was the reports of 15-*cis*-β-carotene in the shoulder following all-*trans*-β-carotene when employing monomeric C_{18} columns. It was later found that 13-*cis*-β-carotene and 9-*cis*-β-carotene elute at the same retention time and thus, these isomers may have been present in the samples [78]. Even in systems that separate the main *cis* isomers of β-carotene, some of the minor isomers are not completely resolved [78]. Incomplete resolution is apparent with other carotenoids as well. Fig. 5 illustrates the detection of several *cis* lycopene peaks but also the lack of baseline separation for this difficult analysis [106].

Because there is a lack of commercial standards for geometrical isomers, care needs to be taken when identifying carotenoid isomers. UV–visible spectral information alone can not be used for positive identification, since coeluting isomers can lead to spectra representing a mixture of isomers. Additional means of identification should be used in interpreting HPLC separations, such as mass spectral [126] and ultimately NMR investigation, as was used by some investigators [71,104]. In spite of this, numerous papers have relied on only UV–visible spectra for their identification of carotenoids.

5.3. Current understanding of cis/trans carotenoid isomers

The main *cis* isomers detected in tissues and carotenoid preparations are the 13-*cis* and 9-*cis* forms. Some 15-*cis* carotenoids are found in larger quantities but most tissues contain only small amounts. Other forms, especially di-*cis* isomers have been detected in small amounts for some of the carotenoids (mainly β-carotene). The major causes of the occurrence of *cis* isomers in foods appears to be the result of their formation with heat processing. It appears *cis* isomers circulate in the plasma and are deposited in human tissues. It has been shown that *cis* isomers generally have lower provitamin A activity than the all-*trans* form. On the other hand, new studies indicate that 9-*cis*-β-carotene is more readily deposited in tissues relative to all-*trans*-β-carotene for several animals [111]. Thus, this isomer may play a role in increasing an animals ability to absorb larger amounts of carotenoids. However, it is interesting to note that 9-*cis* isomer forms of several carotenoids do not appear to be found in serum samples, but more often deposited in tissues. As has been postulated for all-*trans*-β-carotene, whether the isomeric forms have a protective effect against oxidation *in vivo* has not been determined. More studies into the metabolism and physiological consequences of *cis/trans* isomers in the diet are needed. However, further chromatographic method developments for the detection and quantitation of *cis/trans* carotenoid isomers will be required.

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Review

Chromatographic methods for the analysis of heterocyclic amine food mutagens/carcinogens

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ABSTRACT

A series of potent heterocyclic amines that are mutagenic and carcinogenic have been discovered that are formed in some heated foods, most notably, meats derived from muscle. Determining the heterocyclic amine content in foods and food products is required for toxicological research, industry quality control, and possibly in the future, regulatory control. The contents of food needs to be determined using reliable analytical techniques.

Since heterocyclic amines are present in foods at ng/g levels, a variety of liquid–liquid or solid–phase purification techniques are required, followed by gas or high-performance liquid chromatography. Peak detection has been successful using UV, fluorescence, and mass spectrometric detection, and biological activity using the Ames/*Salmonella* test. The low levels present require that chromatographic efficiency, and both detector sensitivity and selectivity be optimized. The cartridge solid-phase extraction and high-performance liquid chromatography method have been used to measure the known food-derived heterocyclic amines for several types of food, and to the authors knowledge, this is the only method undergoing intralaboratory comparison and validation.

Our analysis of the literature shows that chromatographic analysis of the heterocyclic amines by high-performance liquid chromatography or gas chromatography (with derivatization) is satisfactory for heterocyclic amine analysis in foods although the methods are just now being optimized for routine use. The biggest improvements in speed and accuracy will probably come from improved extraction methods as analysis of complex food samples for heterocyclic amines will always be a challenge.

CONTENTS

1. INTRODUCTION

Diet has long been associated with varying cancer rates in human populations, yet the causes of the observed variation in cancer patterns have not been adequately explained [1]. Since 1980, a series of potent heterocyclic amines that are mutagenic and carcinogenic have been discovered. These substances are formed in some heated foods, most notably, muscle meats. The observed higher cancer rates in those populations consuming a western diet which is high in meat has been attributed to high fat or low fiber. Heterocyclic amines are also associated with meat, and unlike fat or fiber, heterocyclic amines have the necessary genotoxic reactivity to lead to the initiating events in the cancer process.

The heterocyclic amines are carcinogens in mice, rats, and monkeys [2,3]. Several of these compounds are among the most potent mutagenic substances ever tested in the Ames/*Salmonella* mutagenicity test [4]. These compounds are also mutagenic in mammalian cells in culture and can cause chromosomal changes in these cells and in mice [5].

These heterocyclic amines are natural products formed from the heating of proteinaceous foods, most notably, muscle meats. Precursors for the formation of these mutagenic and carcinogenic compounds have been shown to be amino acids, such as phenylalanine, threonine and alanine; creatine or creatinine; and sugars [6]. Cooking temperature and time are also important determinants in the formation of these compounds in foods. These variables introduced from the formation process create a large range of possible heterocyclic amine concentrations in foods, requiring the analysis of a large number of samples to determine the heterocyclic amine content in the human diet.

2. ANALYTICAL CONSIDERATIONS

2.1. Goals of analytical method development

The finding of potent mutagenic activity in cooked foods required chromatographic work to isolate and structurally identify the chemicals responsible. Now that many of the chemicals have been identified and synthesized and shown to be animal carcinogens, the next research goals are to quantify the amounts present in foods. Determining the importance of the heterocyclic amines in human health requires accurate exposure (consumption) data with information from comprehensive epidemiology studies. This information can be combined with the rat, mouse and monkey carcinogenic potency assessment for calculation of risk.

Thus far, heterocyclic amines in foods are not regulated by government agencies, although their carcinogenic potency or the amounts detected, in many cases exceed those of many regulated compounds such as chlorinated compounds, pesticides and aflatoxins [7].

The scientific goal in the analysis of these specific carcinogens in foods is to determine the amounts and types of heterocyclic amines present. Besides the need for the determination of the human dose of these heterocyclic amines, the analysis methods could also be used to help devise cooking methods and food preparation strategies to reduce the formation of these compounds in foods. In addition, government or industrial regulation of heterocyclic amines in food may be warranted in the future, and practical methods for food analysis must be available if needed.

From a practical standpoint, a chromatographic analysis method for heterocyclic amines in foods must be accurate and reproducible. Effort must be made to have methods low enough in cost to be used for many, perhaps hundreds of samples per

year. Acceptable methods need to be low in operator and instrument time, instrument cost, and solvent and reagent cost. The method should also minimize waste generation.

2.2 Chemical characteristics of the heterocyclic amine compounds

The heterocyclic amines found in foods have stable multi-ring aromatic structures and all have an exocyclic amino group. Structures of those commonly detected in foods are shown in Fig. 1. From their structures, liquid chromatography and gas chromatography (with derivatization) appear to be suitable chromatographic analysis methods. All of the heterocyclic amines have characteristic UV spectra and high extinction coefficients, some of the compounds fluoresce and all can be electrochemically oxidized making UV absorbance, fluorescence or electrochemical detection suitable methods.

For gas chromatography (GC), detection by electron-capture or nitrogen–phosphorous thermionic detectors is possible. The aromatic structures of these heterocyclic amines give little fragmentation and therefore show large base peaks, making mass spectrometry (MS) a good detection method following either GC or high-performance liquid chromatographic (HPLC) separations.

2.3. Scope of the analytical problems

The are several factors that make the analysis of heterocyclic amines from foods a difficult problem. Heterocyclic amines are present in foods at low ng/g levels [8]. The low levels require that chromatographic efficiency and both detector sensitivity and selectivity be optimized.

Several of the heterocyclic amines are formed under the same reaction conditions, so the number of compounds of interest requires that the extraction, chromatographic separation and detection be general enough to detect several of the heterocyclic amines per chromatographic experiment.

The complexity and diversity of food sample types needing to be analyzed requires a rugged method not affected by the sample matrix. The extraction methods used are an integral part of the chromatographic analysis because the extractions are suited to the requirements of the chromatography and detection. The sample extraction and preparation methods will be discussed in detail for each chromatographic technique presented in this paper.

3. CHROMATOGRAPHIC ANALYSIS METHODS

3.1. HPLC with detection by bacterial mutagenesis

In the late 1970s the detection of bacterial mutagens in cooked meats led to efforts to isolate the

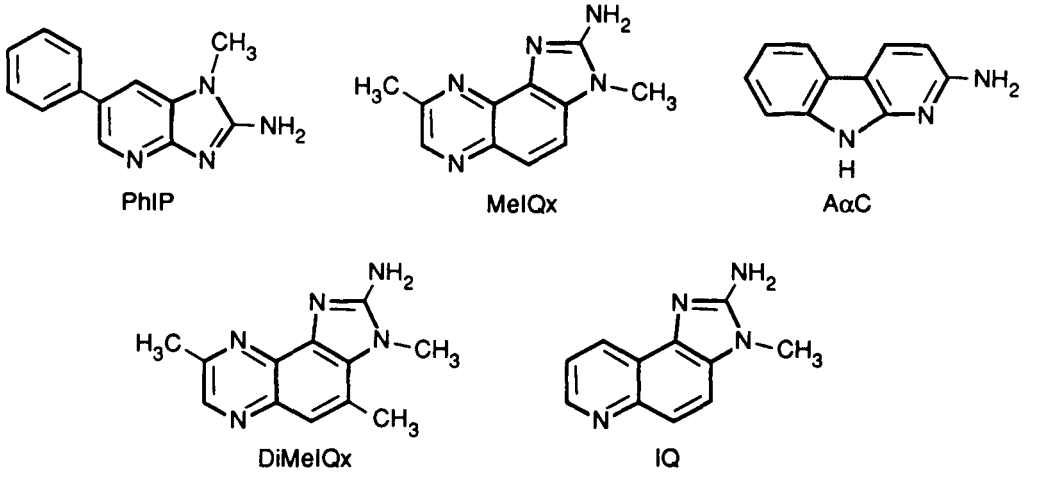

Fig. 1. Structures of five heterocyclic amines commonly found in cooked food. PhIP = 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MeIQx = 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; AαC = 2-amino-9H-pyrido[2,3-*b*]indole; DiMeIQx = 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; IQ = 2-amino-3-methylimidazo[4,5-*f*]quinoline.

mutagenic chemicals using a variety of extraction methods followed by several HPLC steps. Working on a preparative scale, and with the goal of purifying microgram amounts of the mutagenic chemicals, samples were extracted in acid or organic solvents. Further purification was done using base extractions and open column adsorption or chromatography steps. These workers used the mutagenic activity of collected fractions at each step to guide the purification of the heterocyclic amines for structural determination [9–11]. These studies also gave an estimate of the quantity of the chemical in the original food, but extraction losses could not be accurately determined.

Because the bacterial mutation test is not affected by the hundreds of non-mutagenic chemicals also present following separation by HPLC, complex samples could be characterized by collecting fractions and testing each fraction for mutagenic activity. The mutagenic activity profile of collected fractions from HPLC separations was used to determine that the minimum number of mutagenic compounds formed in cooked beef was six [8]. Mutagenic activity profiles of beef cooked at different temperatures showed that mutagenic compounds with similar retention times and relative amounts were formed despite the generation of tenfold more mutagenic activity with the higher cooking temperatures [12].

Fig. 2 shows the HPLC separation of equal amounts of mutagenic activity from the extract of beef, chicken and fish, all ground and cooked as patties at 220°C for 10 min per side. The samples show a similar pattern of mutagenic activity in peak size and retention time despite the differences in meat source and the large difference in total mutagenic activity. The samples have a ratio of mutagenic acitivity of 1:5:8 for fish, chicken and beef, respectively.

The effect of mutagen-increasing additives before cooking was also investigated using the mutagenic activity of collected HPLC fractions. It was determined that creatine was a precursor for almost all of the HPLC separable mutagenic compounds in fried-ground beef and chicken [13]. The effect of the addition of individual amino acids, and creatine to ground pork before frying was noted by Övervik *et al.* [14] using the HPLC–bacterial mutagenesis analysis method.

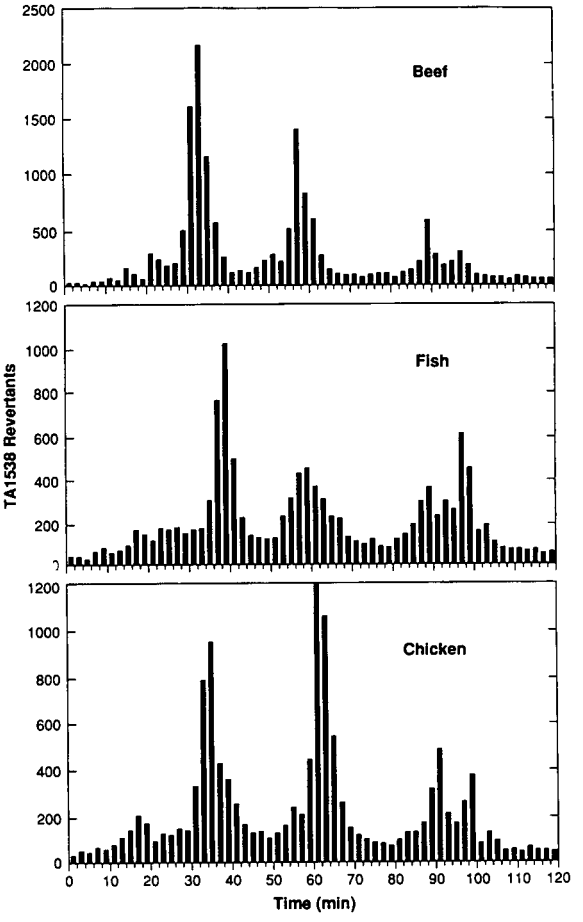

Fig. 2. Mutagenic activity profiles of collected fractions from the HPLC separation of ground-fried beef, fish and chicken. Elution from a PRP-1 styrene–divinylbenzene column was with 15% acetonitrile, 0.1% diethylamine in water for 70 min, with a gradient to 100% acetonitrile at 140 min. Equal amounts of mutagenic activity were injected for each sample.

The HPLC–bacterial mutagenesis method is useful for characterizing samples containing unknown mutagenic chemicals for which no standard compounds are available. Quantitation is crude, but it is useful for sample-to-sample comparisons. Chromatographic coelution experiments using the analysis of collected fractions can also be done despite the complexity of the samples as determined by UV absorbance.

This method is very labor intensive and has a slow sample throughput since samples must be collected and tested for mutagenic activity following

each separation. Quantitation is limited since many sample-handling steps are needed. The range of mutagenic activity per microgram of the heterocyclic amines reported in the literature spans several orders of magnitude and illustrates the problem in estimating of the mass of the mutagenic chemicals from measurement of mutagenic activity.

3.2. Cartridge solid-phase extraction–HPLC

Analyzing heterocyclic aromatic amines at nanogram levels with HPLC and ultraviolet or fluorescence detection requires chromatograms free from interfering peaks. Co-extracted matrix components influence analyte detection limits more than does the absolute detector sensitivity. The sample work-up therefore, is the most critical part of heterocyclic amine analysis. Work at Nestec [15–17] focused on developing practical solid-phase extraction (SPE) based procedures allowing high sample throughput and high analytical sensitivity in food analysis. SPE refers to procedures using disposable cartridges typically containing 100 to 500 mg of a solid, often silica-based, sorbent. Advantages of SPE are considerable simplification and speeding up complicated sample preparation prior to chromatographic analysis.

Food samples such as fried meat or fish require an initial extraction. Diatomaceous earth (Kieselgur, Extrelut), a sand-like porous material is a very practical carrier for extracting all known heterocyclic amines from solid or liquid foods. The inert diatomaceous earth carrier allows efficient and rapid organic solvent extraction without any risk of emulsion formation, a very common problem when extracting food. Fig. 3 shows the extraction profile of radiolabeled 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), indicating that more than 70% of MeIQx and 50% of PhIP are recovered with 40 ml dichloromethane. The crude extract contains basic and neutral substances, however, sensitive analysis of low heterocyclic amine levels in such an extract is not possible due to co-extracted interfering substances. Therefore, additional sample preparation procedures using solid-phase-extraction steps were developed.

Sequential application of several SPE media with different selectivities reduces matrix background as

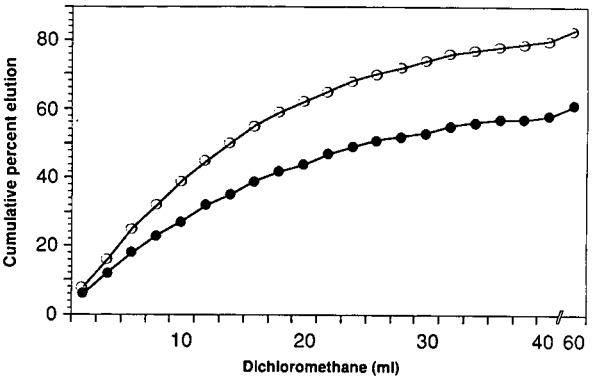

Fig. 3. Extraction of MeIQx and PhIP from fried meat with Kieselgur carrier. Fried meat samples were spiked with [^{14}C]MeIQx or [^{3}H]PhIP and loaded on Extrelut-20 colums as described [15]. During extraction with dichloromethane 20 fractions of 2 ml and one 20-ml fraction were collected. The graph shows the cumulative recovery of both [^{14}C]MeIQx (○) and [^{3}H]PhIP (●) as a function of the extraction volume.

a means to lower analyte detection limits but with the knowledge that multiple purification steps increase the time required for the sample preparation and can introduce sources of errors. Coupling SPE cartridges to "tandems" eliminates time-consuming sample handling steps such as evaporations and resuspensions. The key finding leading to the development of this tandem extraction method was that cation-exchange resins retain heterocyclic amines in dichloromethane which is used for the initial sample extraction with diatomaceous earth.

Cartridge extraction of food products using coupled diatomaceous earth and propylsulfonic acid silica (PRS) cartridges efficiently concentrates basic compounds but PRS also retains other dichloromethane-soluble polar compounds which may interfere with the amines during HPLC. Using the ion-exchange properties of PRS cartridges, a simple procedure to remove most of the unwanted co-extracted interfering peaks was developed. Drying the PRS cartridge with the adsorbed basic extract followed by reequilibration with 0.1 *M* hydrochloric acid initiates ion-exchange binding between heterocyclic amines and the resin. Two simple elution steps subsequently separate heterocyclic amines into two groups, the carboline derivatives and the 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-type compounds. Concentration of these eluates is con-

veniently achieved on a C_{18} silica cartridge. The heterocyclic amines can be eluted and conveniently concentrated by passing over a C_{18} silica cartridge, rather than by rotary evaporation, and finally eluted into autosampler vials with a methanol-ammonia solution mixture [15].

Despite being a multi-step procedure, the diatomaceous earth–PRS method extraction and clean-up does not included any off-line transfers requiring rotary evaporator concentration and resuspension and is well suited for application in routine quality control. Quantitative determinations are done by HPLC with multichannel UV and fluorescence detection. The levels detected were usually in the low ng per gram range with the most abundant compounds found in food samples being MeIQx, PhIP and 2-amino-9H-pyrido[2,3-*b*]indole (AαC) [16].

3.2.1. HPLC

Baseline separation of all heterocyclic amines was a prerequisite for the multi-compound analysis method. The TSK gel ODS80TM (TosoHaas, Montgomeryville, PA, USA) column showed superior peak symmetry and separation efficiency as compared to other HPLC columns tested. Binary mobile phase gradients with acidic buffer (between pH 3 and 4) and acetonitrile gave good peak shapes, but above pH 3.2, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) co-eluted, and below pH 3.5, 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) and PhIP were not baseline separated. They could be resolved though, using a ternary gradient including pH switching from pH 3.2 to pH 3.6 during the run as shown in Fig. 4 [16].

3.2.2. Quantification model

The extraction efficiency of heterocyclic amines following a purification procedure as outlined above is less than one hundred percent. Using a single internal standard added to each sample at the beginning of the extraction is a possible way to estimate analyte losses [15]. This is not ideal because heterocyclic amines include several classes of compounds which are extracted with different efficiency. Even within a group of compounds significant variations may exist as shown for MeIQx and PhIP in Fig. 3. Moreover, the extraction efficiency of heterocyclic

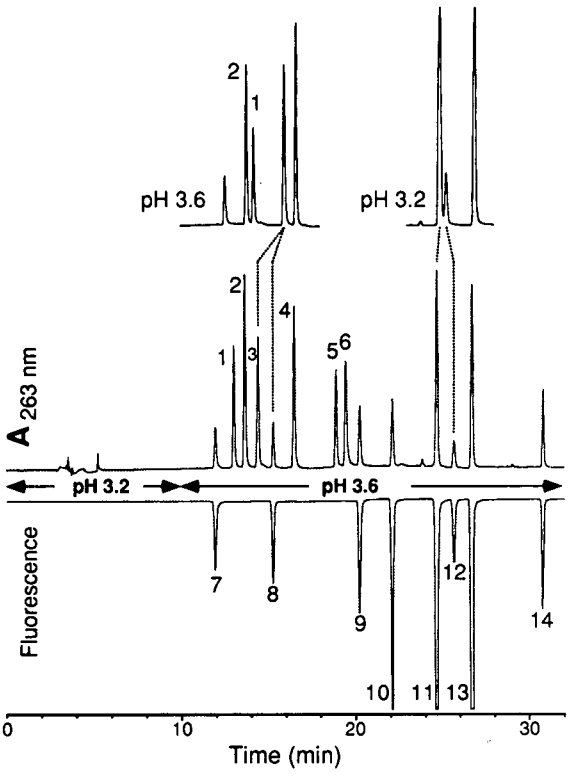

Fig. 4. Separation of heterocyclic amine reference standards. A ternary mobile phase system was used to separate 14 heterocyclic amines on a Toyo Soda TSK gel ODS80TM column (250 mm × 4.6 mm I.D.). A = Triethylamine phosphate (TEAP) 0.01 *M* pH 3.2; B = TEAP 0.01 *M* pH 3.6; C = acetonitrile. Linear gradient: 5–15% C in A from 0–10 min.; 15–25% C in B from 10–20 min; 25–55% C in B from 20–30 min. Peaks are identified as follows (in parentheses excitation/emission wavelengths used for fluorescence detection): 1 = IQ; 2 = 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx); 3 = MeIQ; 4 = MeIQx; 5 = 7,8-DiMeIQx; 6 = DiMeIQx; 7 = 2-amino[1,2*a*:3′,2′-*d*]imidazole (Glu-P-2) (360/450); 8 = Glu-P-1 (360/450); 9 = norharman (300/440); 10 = harman (300/440); 11 = Trp-P-2 (263/410); 12 = PhIP (315/390); 13 = 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole (Trp-P-1) (263/410); 14 = AαC (335/410). A 20-μl volume of a mixture of *ca.* 5 ng per compound was injected.

amines is somewhat dependent on the surrounding matrix and, therefore, difficult to predict precisely for different foods. Standard addition quantification is the method of choice for assessing individual recovery rates for each analyte. Triplicate or quadruplicate extractions with one or two samples spiked with a mixture of reference standards to obtain sets of concentration data for each analyte is

adequate. Linear regression analysis using the added concentration of reference standard (x) as dependent variable and the measured concentration (y) as independent variable enables calculation of individual extraction efficiencies and, therefore, to make accurate corrections of incomplete analyte recovery. Standard errors of slope and intercept give a good estimation of the achieved analytical precision. The necessity of performing replicate extractions may also be seen as a handicap lowering the sample throughput. Stable isotope dilution quantification using selected ion monitoring (SIM) LC–MS or GC–MS would be more economic in terms of sample extractions.

3.2.3. Validation, detection limits

Checking the reproducibility of any method by independent laboratories is crucial if the method is to be used routinely. In a 1991 study, four independent laboratories in the USA and Europe used the diatomaceous earth–PRS tandem extraction method to determine heterocyclic amine content in a meat extract. Analyte recoveries as observed in the four laboratories ranged from 41 to 85% for MeIQx, and from 43 to 83% for 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx). Recovery correction was done using the standard addition quantification model and lowered the interlaboratory variations almost threefold. A similar validation study will be completed in Europe in 1992.

The detection limits of heterocyclic amines purified through diatomaceous earth–PRS tandem extraction were variable and depended on the food sample investigated. With meat extracts or fried meat not processed at temperatures above about 250°C, detection limits may be as low as 1 ng/g using UV detection and below 1 ng/g with fluorescence detection. The question of limits of detection are crucial since many food products investigated to date did not show any detectable levels of heterocyclic amines. Limits of detection can be verified individually using low level spiking as shown in Fig. 5 for a commercial precooked meat product. Samples processed at high temperatures, *e.g.*, by grilling or flame-broiling, usually showed higher levels of interfering peaks. The detection limits then worsened to about 5 ng/g of food.

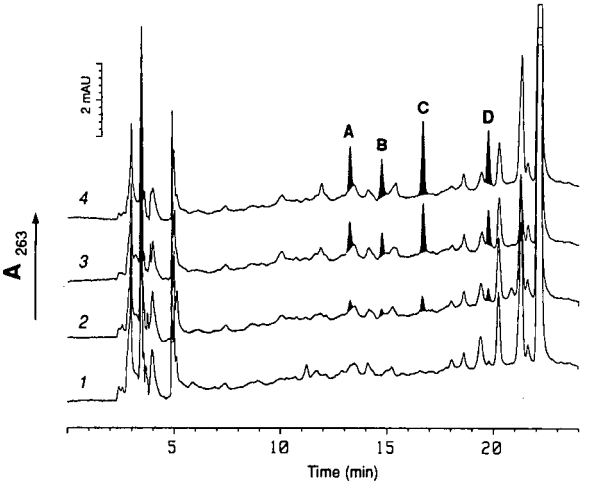

Fig. 5. Evaluation of heterocyclic amine detection limits through spiking with low levels of reference standards. A meat-containing industrially-prepared food product was extracted in quadruplicate using the Extrelut–PRS tandem extraction method and each extract analyzed by HPLC. The figure shows chromatograms of the four polar extracts (0.8 gram equivalents injected). Sample 1 was unspiked, whereas samples 2–4 were spiked with 0.25, 0.75 and 1.2 ng per gram heterocyclic amine reference standards IQ (A), MeIQ (B), MeIQx (C) and 4,8-DiMeIQx (D). Chromatographic conditions as in Fig. 4.

3.2.4. Peak identity confirmation

Peak confirmation is a crucial problem when working with such low levels of heterocyclic amines since co-elution with other co-extracted compounds can occur. The most convenient and accessible instrument to identify heterocyclic amines on-line during an HPLC separation is the UV photodiode array detector. Commercial software included with most instruments allows the recording of interpretable UV spectra even at low nanogram levels using background subtraction. A photodiode array detection system efficiently prevents false peak identifications as shown in Fig. 6 for a peak with the retention time of Trp-P-2 but not having the proper UV spectrum.

3.2.5. Solid-phase extraction with TSK CM650 for more complex samples

Some foods showed increased levels of chromatographic interferences, such as high-temperature processed meat and fish (see Fig. 7), and also some industrial flavorings, (so called process flavors), and, therefore, required a more thorough sample clean-up [14].

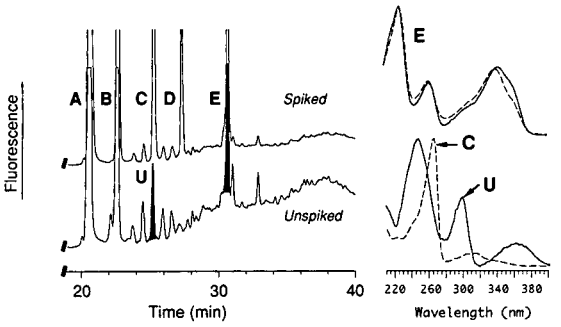

Fig. 6. Peak identification of heterocyclic amines in non-polar extracts of barbecued hamburgers. The left side shows expanded chromatograms of extracts from both spiked (0.8 gram equivalents injected) and unspiked (2 gram equivalents injected) barbecued hamburger samples. Heterocyclic amines identified through retention time matching in the unspiked sample included norharman (A), harman (B), Trp-P-2 (C/U), Trp-P-1 (D), and amino-α-carboline (E). However, as shown on the right with the aid of on-line recorded UV spectra the identification of Trp-P-2 (U) had to be rejected. Chromatographic conditions as in Fig. 4.

A weak cation-exchange material was used as a sorbent with different selectivity than used in the previous purification steps. TSK CM650 has superior affinity and selectivity for heterocyclic amines

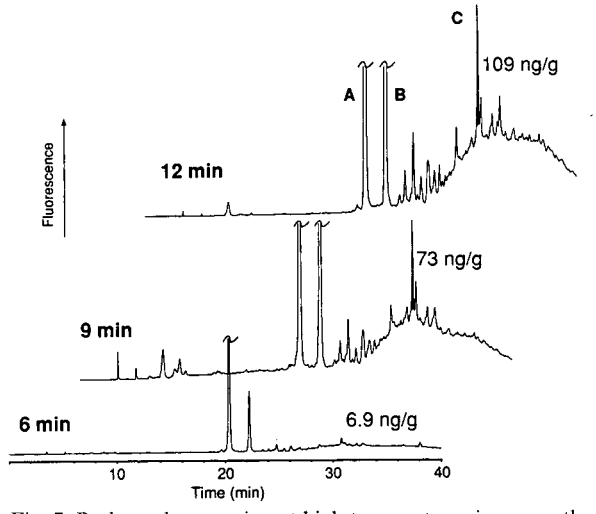

Fig. 7. Prolonged processing at high temperatures increases the level of chromatographic interferences. The graph shows non-polar extract chromatograms from salmon barbecued for 6, 9 or 12 min per side at 270°C. Identified heterocyclic amines include norharman (A), harman (B) and amino-α-carboline (C). Starting at 9 min processing time a dramatic increase in interference levels was clearly visible. Chromatographic conditions as in Fig. 4.

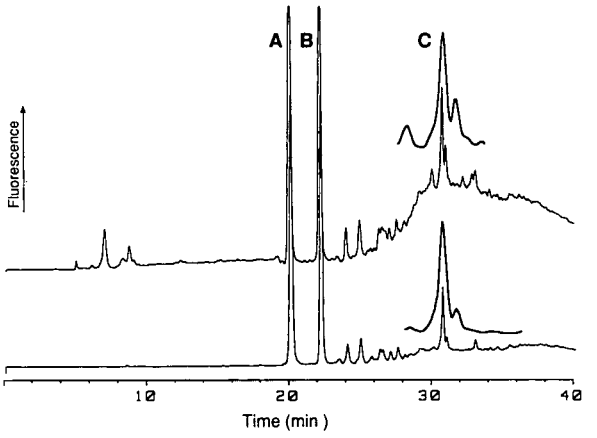

Fig. 8. Solid-phase extraction with TSK CM650 resin lowers chromatographic interferences. Both traces show chromatograms of a non-polar extract of salmon slices barbecued for 12 min per side (0.7 gram equivalents injected). The three major peaks are norharman (A), harman (B) and amino-α-carboline (C). The upper trace is from an extract purified by tandem extraction with Extrelut–PRS, the lower trace was obtained after additional solid-phase extraction using 250 μl TSK CM650 resin and illustrates the background removal effect. Amino-α-carboline peaks are enlarged. Chromatographic conditions as in Fig. 4.

compared to silica-based weak cation-exchange resins. The recovery of analytes from the TSK CM650 resin is close to 90% and its selectivity is impressive. TSK CM650 clean-up was applied to purify extracts of process flavors [17] and high-temperature-treated meat and fish. Interfering peaks in extracts prepared by diatomaceous earth–PRS extraction were significantly lowered (Fig. 8) using this treatment.

The cartridge solid-phase extraction–HPLC method has been used on a variety of samples including meat extracts, cooked meats and process flavors. Although complex samples are the subject of ongoing research, this method is currently being tested in multiple-laboratory collaborative studies which is an important step toward its widespread use.

3.3. Derivatization GC–MS

The most sensitive approach for heterocyclic amine analysis is that devised by Murray *et al.* [18]. Using cooked meat samples spiked with heavy-isotope-labeled standards, samples are dissolved in di-

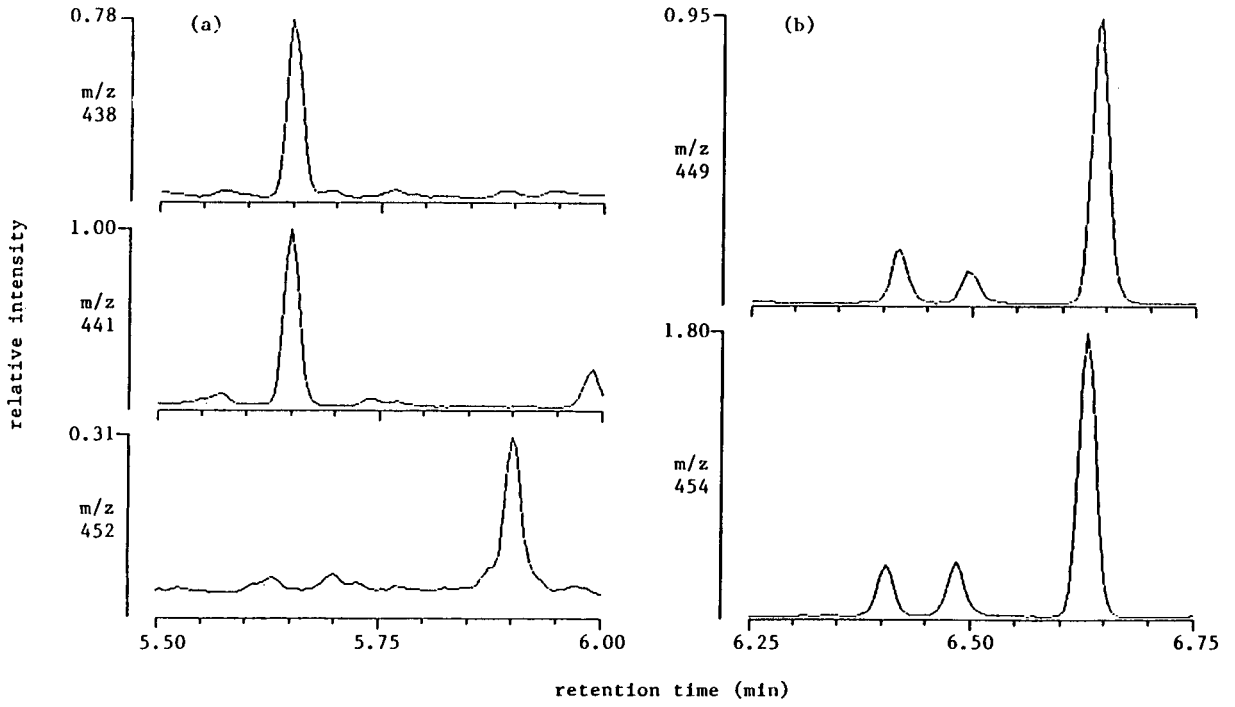

Fig. 9. Selected ion monitoring traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in fried beef. Retention times of the dibistrifluoromethylbenzyl derivatives of MeIQx (*m/z* 438) and [^{13}C, $^{15}N_2$]MeIQx (*m/z* 441) are 5.65 min, of DiMeIQx (*m/z* 452) 5.90 min, and of PhIP (*m/z* 449) and [$^{2}H_5$]PhIP (*m/z* 454) 6.65 min.

lute HCl, washed with dichloromethane to remove oils and fats and then extracted into ethyl acetate after pH adjustment to an alkaline condition.

Dried extracts are then derivatized with 3,5-bistrifluoromethylbenzyl bromide at room temperature, washed with hexane and extracted with ethyl acetate. The total yield for these extraction steps is reported to be about 40% as determined by radioactivity measurements with [^{14}C]MeIQx [18].

Analysis is by GC–electron-capture negative ion chemical ionization MS. Recovery for extraction and derivatization can be calculated from the internal standard. The specificity of single ion monitoring does not require the degree of sample purification that is needed for the LC detectors.

Analysis of derivatized heterocyclic amines uses the high chromatographic efficiency of capillary GC. The chemical ionization and negative ion detection give a reported 1 pg detection limit using SIM.

Fig. 9 shows the results of the analysis of MeIQx, DiMeIQx and PhIP in fried beef by Murray *et al.* Monitoring of appropriate masses yields reasonably clean traces. Calculated results show 3.5 ng MeIQx, 1.2 ng DiMeIQx and 20.3 ng PhIP per gram of cooked beef [19].

Fig. 10 shows analysis of a beef stock cube. MeIQx, DiMeIQx and PhIP were detected at 0.6, 0.3 and 0.3 ng/g, respectively. The limits of detection for the complete liquid–liquid extraction–derivatization GC–MS assay of fried meat and beef stock cubes are 0.05, 0.1 and 0.2 ng/g for MeIQx, DiMeIQx and PhIP, respectively [19].

The sensitivity of this method has also been shown in the analysis of human urine in the determination of MeIQx with a detection limit of 5 pg/ml of urine. About 5% of the MeIQx ingested in beef hamburgers was recovered unchanged in urine [20].

Advantages of this method are the use of isotopically labeled internal standards for accurate quantitation and recovery determination in a single chromatographic run, the unequaled sensitivity of

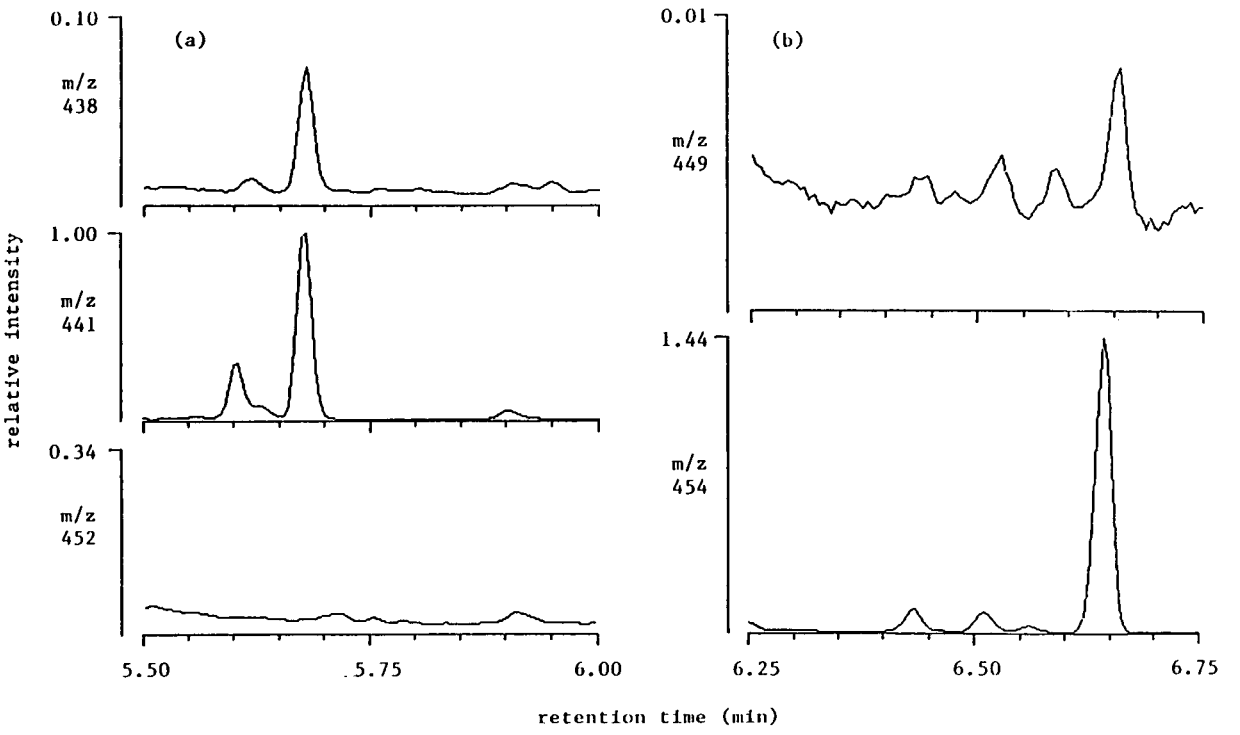

Fig. 10. Selected ion monitoring traces for the analysis of (a) MeIQx and DiMeIQx and (b) PhIP in a beef stock cube. Retention times of the dibistrifluoromethylbenzyl derivatives of MeIQx (*m/z* 438) and [^{13}C,$^{15}N_2$]MeIQx (*m/z* 441) are 5.70 min, of DiMeIQx (*m/z* 452) 5.90 min, and of PhIP (*m/z* 449)) and [$^{2}H_5$]PhIP (*m/z* 454) 6.65 min.

derivatization and negative ion detection, the simplified extraction scheme, and the peak confirmation possibilities using additional ion signals.

Disadvantages include the expensive and specialized instrumentation, the possible uncertainty of the derivatization reactions, especially at low quantities of reactants, and the instability of GC columns used at temperatures from 200 to 320°C. Heavy-isotope-labeled internal standards are needed, although some are available commercially. At present, the specific protocols have been developed only for the analysis of MeIQx, DiMeIQx and PhIP in foods with this method.

3.4. LC–MS

Published methods for LC–MS for the analysis of heterocyclic amines in salmon, sardine and beef were reported by Yamaizumi *et al.* [21] and analysis of beef and beef extracts were reported by Turesky *et al.* [22].

Each procedure required multistep purification with cellulose trisulpho-copper-phthalocyanine (blue cotton) adsorption and acid–base partition. Heavy-isotope-labeled internal standards were used to determine extraction recovery and also for a chromatographic standard. For the published methods, different compounds were examined, IQ and MeIQ for Yamaizumi *et al.*, and MeIQx, DiMeIQx and IQ for Turesky *et al.* Thermospray MS was used and measurements of heterocyclic amines as low as 0.3 ng/g of food were reported by each group.

Fig. 11 shows the thermospray LC–MS analysis of fried beef for IQ, MeIQx and DiMeIQx, and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) by Turesky *et al.* The traces monitored at the appropriate single ion for the (M +

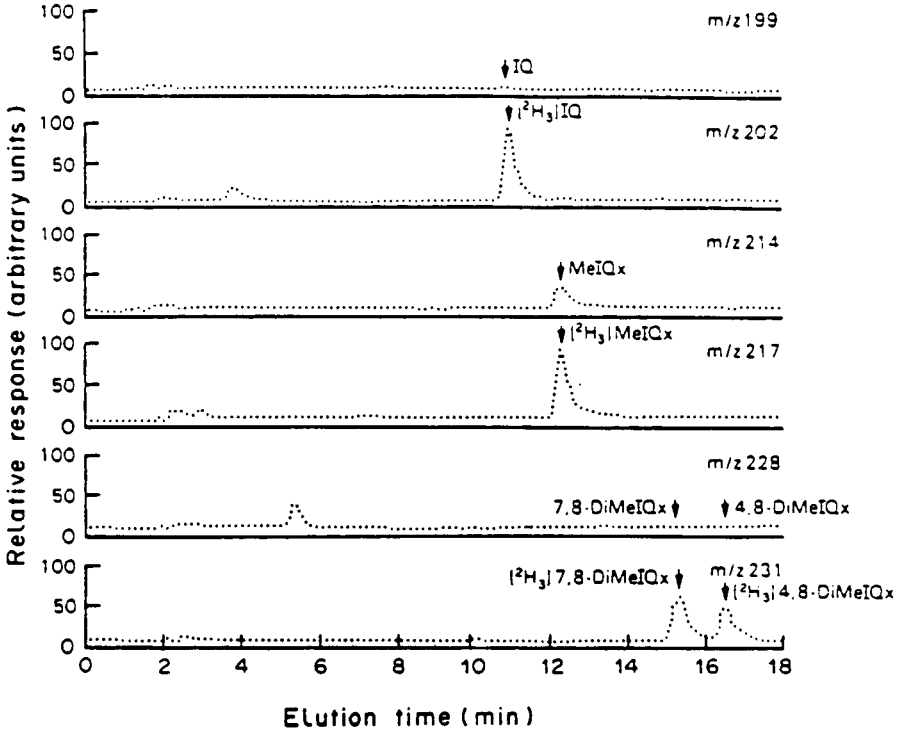

Fig. 11. Selected ion monitoring traces for the thermospray LC–MS analysis of fried meat with isotopically labeled internal standards. Separation was done on a gradient elution of a C_{18} column [22].

$H)^+$ ion are remarkably free of interfering peaks.

LC–MS offers the advantage of column stability compared to GC especially with complex food extracts. MS offers the selectivity of mass detection with confirmation by heavy-isotope-labeled standard coextraction and coelution.

Disadvantages include expensive instrumentation and the need for heavy-isotope-labeled internal standards. The method as published requires time-consuming sample preparation.

3.5. Other purification and detection methods

Electrochemical detection of heterocyclic amines was reported for beef extracts following acid–base partition, cellulose trisulpho-copper-phthalocyanine and silica adsorption [23,24]. Electrochemical detection offers increased detector specificity over UV absorbance detection, although the sensitivities of the two detector types are about the same. Peak confirmation with electrochemical detectors is not possible as it is with photodiode array detectors. Methods analyzing prepared foods with electrochemical detection have not been reported in the peer-reviewed literature, but results of food analysis with electrochemical detection were included in a review [25].

Immunoaffinity chromatography was used for the purification of IQ and MeIQx from heated beef products [26]. Following solubilization, samples were adsorbed on XAD-2 resin, eluted, and applied to affinity columns which had antibodies immobilized on them to IQ or MeIQx. Resulting eluents were chromatographed using HPLC and photodiode array detection. Specific antibodies are needed for each heterocyclic amine, but to determine only one marker compound, immunoaffinity column chromatography offers impressive specificity.

Monoclonal antibodies were developed for the direct analysis of heterocyclic amines in food extracts, but cross-reacting interfering substances made quantitation of heterocyclic amines in these complex samples impractical [27].

4. CONCLUSIONS AND FUTURE NEEDS

It appears that some type of chromatography will always be required for the analysis of heterocyclic amines from foods following rigorous sample clean-up.

The cartridge SPE scheme is a dramatic improvement over the liquid–liquid extractions and the use of large columns filled with XAD resin or cellulose trisulpho-copper-phthalocyanine adsorbent. It is probable that the cartridge extraction scheme could be used successfully with some of the other chromatographic separation and detection procedures described above.

The choice of a method depends on the scientific information needed and complexity of the sample. If only a single marker compound is necessary, extractions can be modified to optimize for a single analyte. For quantitation of all known heterocyclic amines, the general detection advantages of HPLC are required. More concentrated samples like bacterial or food-grade beef extracts could be analyzed by any of the methods presented. Meats and process flavors require extensive sample clean-up for HPLC analysis or the specificity of a MS detector. To detect very low concentrations of analytes (below 1 ng/g), the derivatization GC–MS instrumentation method is best suited.

The amounts of heterocyclic amines in cooked beef or fish published to date show ranges from 0.02–0.36 ng/g for IQ, 0.9 to 8.3 ng/g for MeIQx and 9.7 to 49 ng/g for PhIP [28]. These measurements are all from laboratory-cooked food, the range of amounts in a typical human diet has not been determined.

Validation for the methods discussed in this review should include the determination of the precision, accuracy, and ruggedness as used in other laboratories in comparative studies. To our knowledge, only the cartridge SPE–HPLC method is being used in interlaboratory comparisons. An important criteria for method accuracy is to demonstrate that the results obtained from one method are the same as those determined by a second and independent method. To our knowledge, the comparison has not been done for any of the published methods.

The cartridge SPE method is well developed for 12 heterocyclic amines. Still, new column materials could improve yields or reduce interfering peaks during chromatography, particularly for the more complex foods produced at higher temperatures. Improved columns may help resolve coeluting peaks or increase detector sensitivity for HPLC or GC. And for GC more stable separation columns, particularly those used at higher temperatures, would improve reproducibility.

For all of the analysis methods discussed, improved detector sensitivity or specificity would improve quantitation. The faster and less expensive computers now available for almost any detection system reduce the cost and time needed for data processing especially when large numbers of samples are analyzed.

The variety and complexity of foods and the small amounts of the heterocyclic amines present insures that the analysis of low nanogram levels of these compounds in such a complex matrix such as food will never be simple or inexpensive. Still, great progress has been made in heterocyclic amine analysis and a variety of chromatographic methods have been successfully used. The experience of the chromatographer and the instrumentation on hand will play a large role in the methods chosen. The analysis of foods is just beginning. The validation of the methods presented needs to be made by comparisons of food samples among different laboratories using various methods of analysis.

5. ACKNOWLEDGEMENTS

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Review

Capillary gas chromatography in quality assessment of flavours and fragrances

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ABSTRACT

The latest analytical advances in quality assessment of flavours and fragrances are reviewed, including capillary gas chromatography (cGC)–olfactometry for the identification and sensory evaluation of important odorants by means of odour activity values, aroma extract dilution analysis and stable isotope dilution assay (SIDA). Enantioselective cGC and comparative stable isotope ratio mass spectrometry (IRMS), coupled on-line with cGC, are reported as important tools in the authenticity control of flavours and fragrances. The literature on these topics is reviewed up to the beginning of 1992. The scope and limitations of chirospecific cGC and cGC–IRMS are discussed. The trends and perspectives in the origin control of flavours and fragrances are outlined.

CONTENTS

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1. INTRODUCTION

Flavours and fragrances are complex mixtures of volatile compounds and generally consist of hundreds of substances of different functionalities. They often occur in a great variety of concentrations, from trace levels in the sub-ppb range up to the amounts of the main constituents of essential oils.

Gas chromatography (GC) was introduced at an early stage of flavour research. Flavour chemists were among the pioneers of GC and also, more recently, of capillary GC(cGC) [1,2], as reviewed by Teranishi [3]. Whereas the first era of flavour research was directed to the comprehensive stock-taking of all the volatile constituents of complex flavour and fragrance extracts, the main efforts during the last decade have been focused on constitutional and stereochemical features, chemoreception and sensory relevance and biogenesis and biotechnological synthesis of sensorily active compounds, as reviewed by Schreier and Mosandl [4].

The state of the art in flavour research has been excellently reviewed in a series of papers on "Advances in aroma analysis" and "Progress in analysis of odorous substances" by Werkhoff *et al.* [5,6], including methods of isolation, concentration and separation of volatiles. New methods of chirospecific analysis, preparative techniques in GC and high-performance liquid chromatography (HPLC) and modern methods in structure elucidation were also summarized.

This review is directed to the analytical advances in the quality assessment of flavours and fragances, using capillary gas chromatography–olfactometry (cGC–O) for the identification of important odorants and combined GC techniques for the origin assignment of flavours and fragrances.

2. CAPILLARY GAS CHROMATOGRAPHY–OLFACTOMETRY (cGC–O)

2.1. Odour activity values (OAV)

In the early stages of flavour research, long lists of volatiles from complex matrices were presented [7] but with poor information about the influence on their sensoric relevance to the food investigated [8].

The first approach in determining the contribution of volatile compounds to bread flavour was made by Rothe and co-workers [9,10]. They calculated the ratio of the concentration (c) to the aroma threshold value (a) for some of the volatiles that had been detected in the crumb of wheat bread, and termed the result "aroma value", $A = c/a$. The odour thresholds (the lowest concentrations that can be sensorily recognized) were obtained by tasting the aroma compounds from aqueous solutions.

The "aroma values" (A) were replaced with the novel term "odour value" by Mulders [11] or "odour activity value" (OAV) as a more precise definition by Blank and Grosch [12]. Although the concepts of aroma and odour values have been criticized by Frijters [13], he stated that odour activity values may be used as a conclusive approximation to volatile compounds which significantly contribute to the flavour of a food.

Even if some synergistic and mutual enhancement effects of different aroma-active compounds are known, the contribution of flavour activity to the flavour impression as a whole normally depends on odour unit values of individual substances ($A \geqslant 1$).

2.2. Aroma extract dilution analysis (AEDA)

The human nose often recognizes aroma-active compounds from the GC effluent at the lowest levels that remain inaccessible even by highly sensitive detection techniques. Thus, "GC sniffing" has been developed as a simple and well appreciated method in locating positions of odorants in a gas chromatogram. However, one should realize that GC sniffing strongly depends on the amount of the food from which the flavour extract is isolated, the concentration of the compound analysed and the sample volume which is injected for GC analysis [8]. Therefore, a single GC sniffing run is completely insufficient in the sensory evaluation of flavour compounds from complex extracts. These difficulties had been overcome by sniffing of a dilution series of the original aroma extract from a particular food. Two variations of aroma extract dilution analysis (AEDA) have been developed as systematic approaches to evaluating potent odorants [14–18]. Acree and co-workers [14,15] calculated combined hedonic response measurements (CHARM) on the basis of the duration of the sensory responses which were maintained during GC sniffing of definitely

diluted flavour extracts. CHARM values correspond directly to odour activity values (OAV).

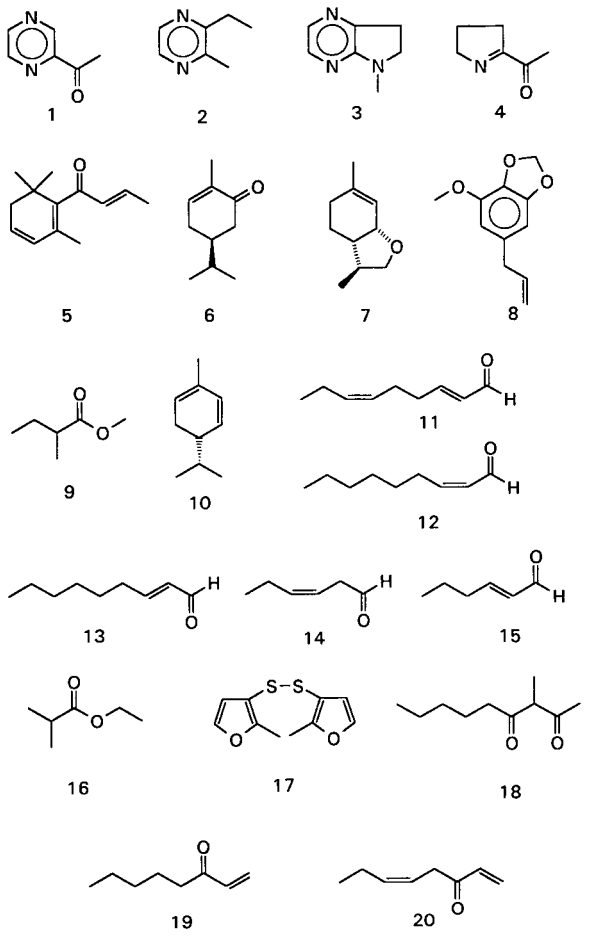
Using the method of Grosch and co-workers [16–18], the aroma extract is diluted stepwise with a solvent until the odour-active region in the GC effluent is no longer detectable. A flavour dilution factor (FD factor) has been defined as the highest dilution at which a compound is still sensorily recognized in the GC effluent. In this way, volatile compounds from an aroma extract are actually ranked according to their odour activity values (OAV).

3. STABLE ISOTOPE DILUTION ASSAY (SIDA)

Reliable quantification of character impact flavour compounds in foods can be a difficult task, because aroma-active compounds with high FD factors often occur in extremely low concentrations in foods, far below their direct analytical detection limit using cGC [19].

More or less laborious steps of enrichment by extraction and separation techniques are therefore essential before determination, and the losses caused by these manipulations have to be corrected with internal standards. However, accurate data can only be expected if the stability and the physical properties of both the compound to be analysed and the internal standards are comparable [20]. The use of stable isotope-labelled internal standards is known as the stable isotope dilution assay (SIDA) and has been widely applied for the determination of trace compounds. Apart from negligible isotope effects, the standards and odorants to be determined are also identical in chemical and physical properties. SIDA was introduced into flavour analysis by Schieberle and Grosch [20], who achieved the accurate determination of acetylpyrazine (**1**), 2-methyl-3-ethylpyrazine (**2**), 5-methyl-5*H*-cyclopenta[*b*]pyrazine (**3**) and 2-acetyl-1-pyrroline (**4**) in bread crust.

The mass spectra of **4** are shown in Fig. 1. Mass chromatography was performed with cGC, coupled to the ion trap detector, running in the chemical ionization (CI) mode. Quantitative analysis in conjunction with OAV values evaluated **4** as the most important and character impact odour compound of the wheat bread crust. It is interesting that the high level of **4** (96 μg/kg; OAV 4800) in the crusts of the wheat breads was the most striking difference between wheat and rye breads [21]. These investigations have been extended, indicating **4** as one of the characteristic roasty/malty flavour notes of fresh popcorn [22] and cooked rice [23].



With regard to the quality assessment of flavour and fragrances, the evaluation of both potent odorants [12,24,26–28] and off-flavour compounds is highly desirable [19,30,31].

SIDA measurements have been developed for the determination of β-damascenone (**5**) in foods and applied to roasted coffee, black tea, honey and beer [24]; (4*S*)-(+)-carvone (**6**) has been described as the character impact compound of dill seed aroma. Dill ether (**7**), myristicin (**8**), methyl 2-methylbutanoate (**9**) and (4*S*)-(+)-α-phellandrene (**10**) were established as the decisive compounds forming the typical odour notes of fresh dill herb [12]. The odour differences between cucumbers and musk melons have

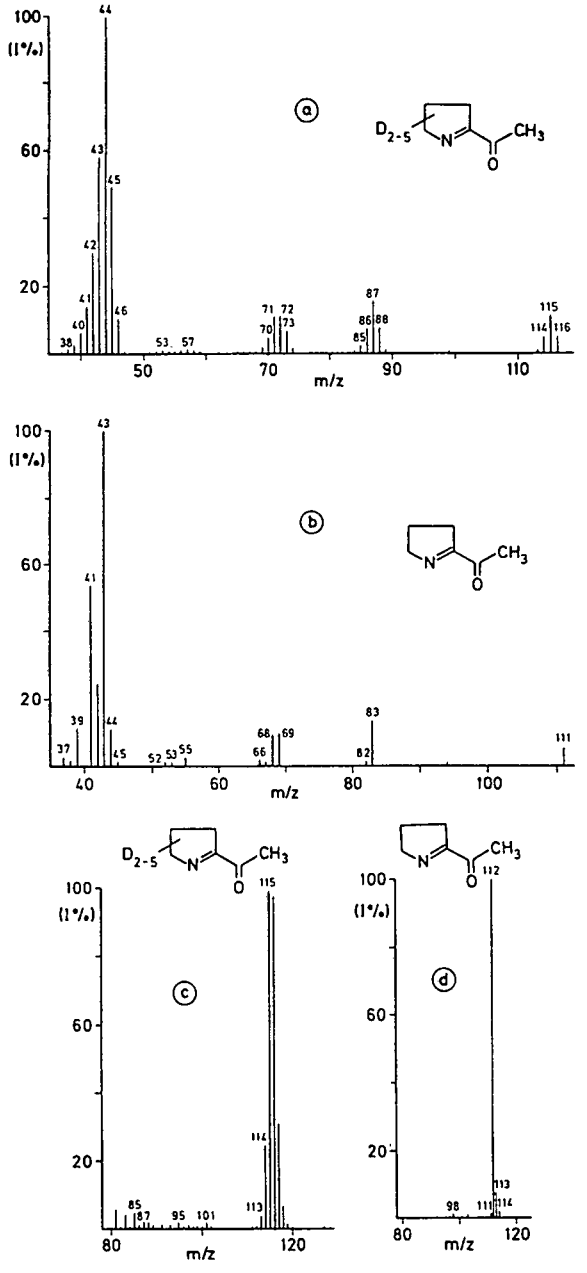


Fig. 1. Mass spectra of (a) [^{2}H]-2-acetyl-1-pyrroline (EI-MS), (b) 2-acetyl-1-pyrroline (EI-MS), (c) [^{2}H]-2-acetyl-1-pyrroline (CI-MS) and (d) 2-acetyl-1-pyrroline (CI-MS). From ref. 20.

been evaluated; identifying (*E,Z*)-2,6-nonadienal (**11**), (*Z*)-2-nonenal (**12**) and (*E*)-2-nonenal (**13**) as the most significant odorants of cucumber, whereas methyl 2-methylbutanoate (**9**), (*Z*)-3-hexenal (**14**), (*E*)-2-hexenal (**15**) and ethyl 2-methyl propanoate (**16**) were responsible for the fruity, green odour of musk melons [26]. Unsaturated alcohols and aldehydes also contribute significantly to the green odour notes of the well appreciated virgin olive oils [27] and the flavour of boiled meat is substantially influenced by sulphur-containing odorants [28].

Fig. 2 compares the EI-MS spectra of bis(2-methyl-3-furyl) disulphide (**17**) with the corresponding bis-2-[$^2H_3$]-derivative as internal standard for SIDA.

AEDA and SIDA techniques have been successfully applied to judge limiting changes in aroma quality during processing or storage of foods [30–32]. In contrast to other plant oils, soya-bean oil is known to be sensitive to daylight. In a daylight-stored sample of soya-bean oil a rapid increase in 3-methyl-2,4-nonadione (**18**) during the first 48 h has been detected [30]. Compound **18** clearly causes the light-induced off-flavour and it is generated by photooxidation of furanoid fatty acids of soya-bean oil [31].

1-Octen-3-one (**19**) and (*E*)-2-nonenal (**13**) are

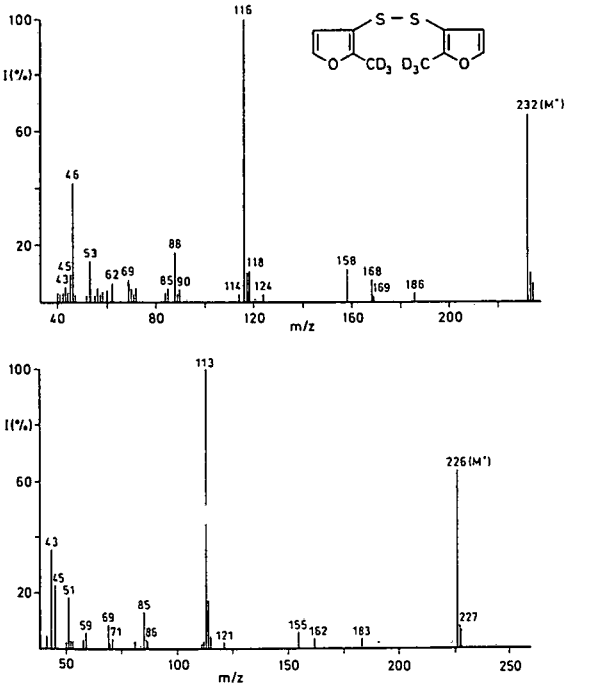


Fig. 2. EI-MS of bis(2-methyl-3-furyl) disulfide (**17**) and labelled derivative (top). From ref. 19.

known as autoxidation products of linoleic, and (*Z*)-1,5-octadien-3-one (**20**) of linolenic acid. As their concentrations may increase significantly during storage of butter oil, **19**, **13** and **20** are recommended as indicator substances for the assessment of the deterioration of butter oil caused by peroxidation [32].

4. GENUINENESS OF FLAVOURS AND FRAGRANCES

Comparative stable isotope ratio analysis (SIRA), also known as isotope ratio mass spectrometry (IRMS), has been described as an important method in the origin assessment of food compounds [49–54]. On the other hand, naturally occurring chiral flavour compounds of high optical purity should be expected, as enzymatic reactions are commonly characterized by a high degree of stereospecificity. Both phenomena, stable isotope discrimination and enantioselectivity, during biosynthesis may serve as the basis to judge the origin of flavour compounds if comprehensive data from authentic sources are available.

Chirospecific cGC methods coupled on-line with SIRA measurements have been reported as highly sophisticated methods in the authenticity control of flavours and fragrances. This is of considerable interest, as naturalness of foods and beverages is in high demand by the customer. Further, legal regulations order the differentiation of natural and non-natural flavouring substances and these methods have also been adopted into quality assurance by the flavour industry.

4.1. Chirality evaluation

Chiral discrimination has been recognized as one of the most important principles in biological activity and also odour perception [33–41]. Besides enantioselective biogenesis, the evaluation of chirality in the origin control of flavours and fragrances has to be discussed with regard to some fundamental conditions.

4.1.1. Chiral resolution and chromatographic behaviour of enantiomers

Resolution (R_s) is defined as the separation of two peaks in terms of their average peak width at half-height (eqn. 1) or at a base width of 4σ (eqn. 2) [57]:

$$R_s = 1.177\left(\frac{\Delta t_R}{w_{h1} + w_{h2}}\right) \quad (1)$$

$$R_s = 2\left(\frac{\Delta t_R}{w_{b1} + w_{b2}}\right) \quad (2)$$

where

Δt_R = absolute difference in retention time of the two peaks 1 and 2;

$w_{h1(2)}$ = width of peak 1 (2) at half-height;

$w_{b1(2)}$ = width of peak 1 (2) at base (4σ).

In the case of enantiomeric pairs, the term chiral resolution (cR_s) is used [55]. As outlined in Table 1, 100% separation occurs if chiral resolution cR_s = 2.50; in practice $cR_s \geqslant 1.50$ (99.73% separation) is defined as a baseline resolution [57]. Thus, optimum chiral resolution ($cR_s \geqslant 1.50$) should be achieved using a suitable chiral stationary phase in enantioselective cGC.

Although the separation factor α has the greatest impact on peak resolution, only in the case of highly resolved enantiomer separation is an accurate determination of high enantiomeric excess (ee values) possible, as shown in Fig. 3 [67].

With the present state of knowledge, the mechanisms of GC enantiomer separation have not been elucidated. Unusual chromatographic behaviour and reversal of the elution order of enantiomers have been observed. Consequently, the usefulness of a given chiral stationary phase and the order of elution of separated enantiomers cannot be predicted. References of definite chirality are essential to identify the separated isomers, no matter whether directly stereoanalysed with chiral stationary phases [61–66,162] or via derivatized stereoisomers [44,62, 66].

TABLE 1

SEPARATION AS A FUNCTION OF cR_s [57]

cR_s	Separation (%)	Δt_R (in σ)
0.5	68.28	2
0.9	92.82	3.6
1.0	95.44	4
1.1	97.22	4.4
1.25	98.76	5
1.50	99.73	6
2.0	99.99	8
2.50	100.00	10

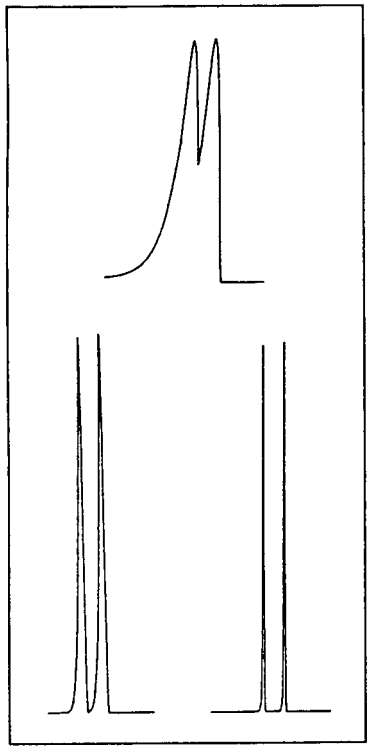


Fig. 3. Chiral separations with identical separation factors (α) and different chiral resolutions (cR_s). From ref. 67.

4.1.2. Sample clean-up

Optimum chiral resolution without any racemization and reliable interpretation of chromatographic behaviour of enantiomers have to be considered as the first targets in chirospecific analysis (section 4.1.1). Even if a universal recommendation on sample clean-up cannot be given, depending on the complexity of samples to be analysed, preseparation procedures of the highest efficiency are needed if a reliable stereodifferentiation is to be achieved.

The demanding challenge is to obtain chiral volatiles of the highest chemical purity, ready for the direct resolution into their mirror images by GC techniques. This ideal may be realized by preseparation techniques in non-chiral media (chiral discriminations excluded), using high-performance thin-layer chromatography (HPTLC) [80,81] or HPLC, off-line [69,71] or on-line [72,73] coupled with enantioselective cGC. In particular, enantioselective multi-dimensional GC (enantio-MDGC) with the combination of a non-chiral precolumn and a chiral main column [74,75] has been demonstrated as a powerful method for the direct stereoanalysis of chiral volatiles without any further clean-up or derivatization procedures [60,68,70,76–79,82–97].

A schematic diagram of enantio-MDGC, well proved in quality assurance and origin control of flavours and fragrances, is shown in Fig. 4. A double-oven system with two independent temperature controls, two flame ionization detectors (D_{M1}, D_{M2}) and a live switching coupling piece is used. With optimum pneumatic adjustment of the MDGC system, definite fractions, eluted from the precolumn, are selectively transferred on to the chiral main column (heart-cutting technique).

First the chromatographic conditions of the chiral main column must be optimized carefully. Capillary columns coated with chiral stationary phases of suitable enantioselectivities are used as main columns. Chiral resolutions are commonly achieved isothermally or by low temperature programming rates, starting at least 20°C below the precolumn temperature. Precolumns are chosen with respect to the versatility of application, to the direct injection of high sample volumes and with respect to the requisite time of analysis.

Under optimized operating conditions, uncoated and deactivated restriction capillaries are installed between the injector and precolumn by means of simple press-fit connectors to reduce the carrier gas velocity within the precolumn. By means of such a column combination, suitable pre- and main columns may be easily exchanged and adapted for optimum efficiency [76].

4.1.3. Detection systems

If optimum chiral resolution and high-efficiency sample clean-up, the first priorities in enantioselective analysis (sections 4.1.1 and 4.1.2), are realized, simple detection systems, such as flame ionization detection (FID) are suitably used. The ideal detector is universal yet selective, sensitive and structurally informative. Mass spectrometry (MS) currently provides the closest approach to this ideal [98]. The combination of multi-dimensional GC with high-resolution MS or mass-selective detectors in the single ion monitoring (SIM) mode is currently the most potent analytical tool in enantioselective analysis of chiral compounds in complex mixtures [77]. Nevertheless, it must be pointed out that the applica-

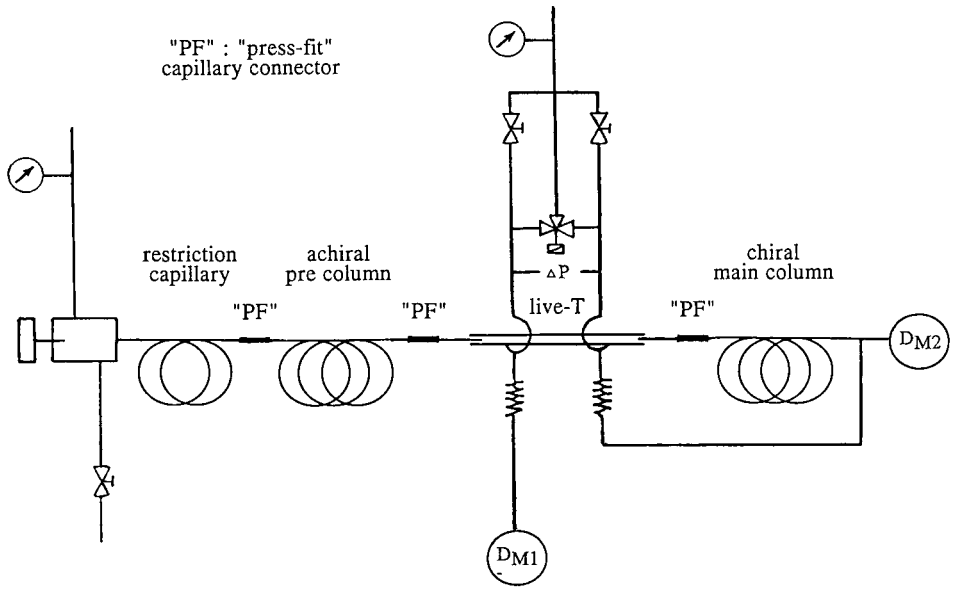


Fig. 4. Schematic diagram of enantio-MDGC according to ref. 76.

tion of structure-specific detection systems such as MS [99] or Fourier transform infrared (FT-IR) spectroscopy [100] cannot save the fundamental challenges to optimum (chiral) resolutions and effective sample clean-up.

The effectiveness of mass selective detection in the SIM (MIM) mode and selected wavelength chromatograms (SWC) in FT-IR detection depends on efficient sample clean-up. In raw extracts the risk of co-eluting substances which cannot be distinguished from chiral volatiles increases with the complexity of flavour and fragrance extracts to be analysed. Hence the direct use of MS (FT-IR) might be seriously limited and the use of such detection techniques in enantioselective cGC without effective preseparations cannot be recommended.

4.1.4. Stereodifferentiation and quantification

The total amount of characteristic flavour constituents and their relative distribution in aroma extracts have been included in official legal assessments for many years, using internal standards for quantification and referring to the merits of comprehensive stock-taking of volatile constituents from complex flavour and fragrance extracts during the first era of flavour research [7]. The measurements are precise and reproducible, but with respect to the validation of flavours and the origin of essential oils their utility is limited [68].

Chirospecific differentiations of optically and sensorially active compounds point out new possibilities in structure–function relationships and also the biogenesis of chiral volatiles [68]. Chiral fruit flavour compounds have been detected with characteristic enantiomeric ratios, defined as fruit-specific enantiomeric distributions [69].

Enantiomeric purity and enantiomeric excess (ee) are the usual terms used in the determination of enantiomers. Enantiomeric purity is defined as the measured ratio (expressed as a percentage) of the detected enantiomers, whereas ee values describe the relative difference of the separated enantiomers (expressed as a percentage). Usually quantifications are given in terms of ee values, but one should note that convincing results can be concluded only for baseline-resolved enantiomers ($cR_s \geqslant 1.50$). Exact calculations of partially resolved mirror images, as frequently occur in the current literature, remain unintelligible in view of significant differences in sensory qualities and odour thresholds of enantiomers [33–41,58–60]. Partially separated enantiomeric pairs ($cR_s = 0.9$–1.50) should be calculated approximatively as the ratio of enantiomers (expressed as a percentage). Approximative quantifica-

tions in terms of enantiomeric ratios are also more conclusive than ee values if concentrations are too low for precise calculations [70].

4.1.5. Limitations

Three types of limitations have to be accepted in chirospecific analysis: racemates of natural origin, generated by non-enzymatic reactions (autoxidation, photooxidation, etc.); racemization during processing or storage of foodstuffs, if structural features of chiral compounds are sensitive; and blending of natural and synthetic chiral flavour compounds. Nevertheless, the systematic evaluation of origin-specific enantiomeric ratios has proved to be a valuable criterion for differentiating natural flavour compounds from those of synthetic origin.

4.1.6. Analysis of individual classes of compounds

The first success in chirospecific flavour analysis was achieved by chromatographic separations of diastereomeric derivatives. In spite of limited sensitivity and frequently laborious work-up conditions, these methods revealed a reliable insight into the enantiomeric distribution of $\gamma(\delta)$-lactones and other chiral fruit flavour compounds [42–48], as reviewed previously [101].

A real breakthrough in chirospecific analysis occurred when enantioselective cGC became available. Three classes of chiral stationary phases are of special interest: amino acid derivatives, chemically bonded to polysiloxanes [102,103]; optically active metal chelates [104]; and modified cyclodextrins [63,67,105–110,162–165]. In particular, since 1988 selectively alkylated/acylated α-, β- and γ-cyclodextrins have been synthesized, serving as chiral stationary phases in enantioselective cGC.

Owing to the high melting points of pure cyclodextrins and many of their derivatives, the chromatographic efficiency of such chiral stationary phases was poor. Improved performance was achieved by König and co-workers [63] and Armstrong *et al.* [107], who introduced cyclodextrin derivatives which are liquid or waxy at room temperature. As an alternative approach, Schurig and co-workers [106,164,165] and Bicchi *et al.* [25] diluted high-melting cyclodextrin derivatives with polysiloxane stationary phases to obtain chiral selectivity below the melting point of the pure cyclodextrin. Dilution of high-melting cyclodextrin derivatives lowers the minimum GC working temperature, but chiral resolution decreases with increasing dilution of the cyclodextrin. In order to prepare columns with maximum chiral selectivity, dilution should be minimal, as demonstrated by Schmarr *et al.* [56].

Although the mechanisms of chiral recognition and molecular inclusion have not been elucidated, modified cyclodextrins have been reported as versatile chiral phases with a wide range of applications. The chiral resolution of many cyclic monoterpenes [67,107,124,127] and investigations of the enantiomeric distribution of α-pinene, β-pinene and limonene from foods, drugs and essential oils have been reported [68,83–85,87]. Also *cis–trans*-α-irones, highly responsible fragrance compounds of iris oil [114,162], filbertone [(*E*)-5-methyl-2-hepten-4-one], the character impact compound of hazel-nuts [163], damascone [151] and geosmin [114] have been separated into their mirror images. Even polar molecules such as 1,2-ketols, 1,2-glycols and free carboxylic acids were successfully stereoanalysed by Mosandl *et al.* [66].

1,3-Oxathiane derivatives. 3-Methylthiohexanol and *cis–trans*-2-methyl-4-propyl-1,3-oxathiane have been reported by Winter *et al.* [111] to play important roles in the delicate flavour of the yellow passion fruit, in spite of their occurrence at extreme trace levels. Using complexation GC, the chirospecific analysis of all four stereoisomers of 2-methyl-4-propyl-1,3-oxathiane was achieved. This was the first direct stereochemical analysis of an essential chiral trace compound from fruit flavours [61]. While first attempts at the chirality evaluation of this potent odorant from yellow passion fruits were described [112], the direct chirospecific analysis of natural 1,3-oxathianes is further under investigation [113]. Modified cyclodextrins [110,114], using enantioselective cGC, serve as promising alternatives (Fig. 5).

γ-Lactones. Chiral γ-lactones are important compounds of many fruits and give strawberries, peaches, apricots and many other fruits their characteristic and distinctive notes [116]. Recently Albrecht and Tressl [117] investigated the biogenetic sequence of γ-decalactone. These results indicate that (*E*)-3,4-epoxydecanoic acid, formed from (*E*)-3-decenoyl-CoA, an intermediate of the β-oxidation of linoleic acid, is the genuine precursor in the biosynthesis of γ-decalactone (Fig. 6.).

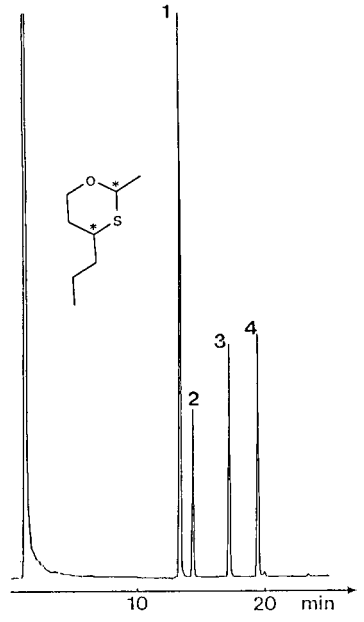


Fig. 5. Chirospecific differentiation of 2-methyl-4-propyl-1,3-oxathiane. Elution order: (1) 2*R*, 4*S* (enriched); (2) 2*S*, 4*R*; (3) 2*R*, 4*R*; (4) 2*S*, 4*S*. From ref. 110.

Owing to their common enzymatic pathways, chiral aroma compounds from fruits and other natural sources should be characterized by origin-specific enantiomeric ratios. Indeed, from freshly harvested strawberries γ-decalactone and γ-dodecalactone have been detected with high optical purity favouring the 4*R*-configured γ-lactones: γ-C_{10} (4*R*; >98% ee), γ-C_{12} (4*R*; >99% ee) [76]. On the other hand, racemic γ-decalactone in aroma-relevant amounts has not yet been observed in fruits. Thus, the detection of racemic γ-decalactone in fruit-containing food indicates the addition of nature-identical γ-C_{10}-lactone. Furthermore, enantioselective MDGC, employing heart-cutting techniques from DB-1701 as the preseparation column on to heptakis (3-O-acetyl-2,6-di-O-pentyl)-β-cyclodextrin as the chiral main column, was described by Mosandl *et al.* [76] as a powerful tool in the direct enantiomer separation of chiral γ-lactones from complex matrices without any further clean-up or derivatization procedures. Comprehensive data on natural γ-lactones from fruits have been reported by Nitz *et al.*

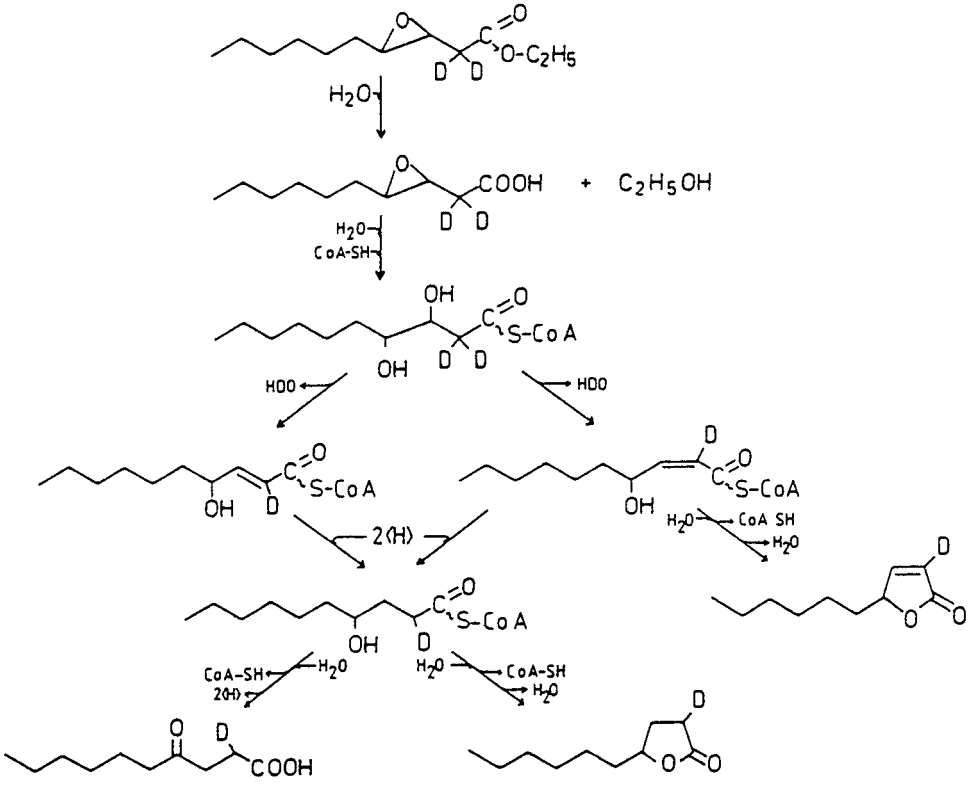


Fig. 6. Proposed transformation of ethyl (*E*)-3,4-epoxydecanoate by cells of *S. odorus*. From ref. 117.

[77], Bernreuther *et al.* [78] and Guichard *et al.* [79] (Fig. 7).

Comparative investigations of γ-lactones from apricots of different varieties and cultivars [118] in connection with corresponding enantioselective analysis [79] indicate some important conclusions: if present at all, odd numbered γ-lactones occur only in trace amounts; the most abundant γ-lactones are the even-numbered homologues γ-C_6, γ-C_8, γ-C_{10} and γ-C_{12}; and the enantiomeric distribution of chiral γ-lactone homologues in fruits has been demonstrated to increase in favour of the 4*R*-configured lactones with increasing length of the alkyl-side chain [77–79,96].

In spite of relatively large amounts of the lowest homologue, γ-C_6 is useless for the origin assessment of flavours, owing to the wide range of ee values detected. A high odour threshold ($a = 13\ 000$ μg/kg) [119] and unspecific odour quality [40] also revealed γ-hexalactone as insignificant to the odour impression of apricots [79]. It is also interesting to note the detection of dihydroactinidiolide as the first γ-lactone racemate of natural origin, generated by photooxidation in ripening apricots [79]. This surprising fact has also been confirmed for dihydroactinidiolide from raspberries [114].

With regard to chirality evaluation as an indicator for the genuineness of natural flavours and fragrances, only chiral volatiles of high optical purity and with characteristic and small ranges of ee values should be validated in relation to their total amounts. Under these conditions, the even-numbered γ-lactones C_8 ($a = 95$ μg/kg), C_{10} ($a = 88$ μg/kg) and C_{12} [119], in particular γ-decalactone, have proved to be useful indicators of naturalness for many fruit flavours and fragrances if their genuine amount occurs in the aroma relevant range (OAV > 1). Of course, chiral compounds occurring in trace amounts and far below their odour activity values in the foods investigated, *e.g.*, γ-decalactone in raspberries (2 μg/kg) and gooseberries [120], have to be neglected.

In this context, it should be mentioned that γ-lactones do not occur as genuine constituents of coconuts [121]. Thus, it seems to be clear that aroma-relevant amounts of γ-lactones in coconut products are of non-natural origin and there is no reason to look for their stereoanalysis in the low-ppb range [120]. On the other hand, chiral δ-lactones from coconuts, in particular δ-octalactone and δ-decalactone, have been evaluated as suitable indicators of naturalness [86].

δ-Lactones. Likewise, chiral δ-lactones are known as characteristic flavour compounds of fruits and dairy products [116]. Their stereodifferentiation was achieved with modified γ-cyclodextrin by König *et*

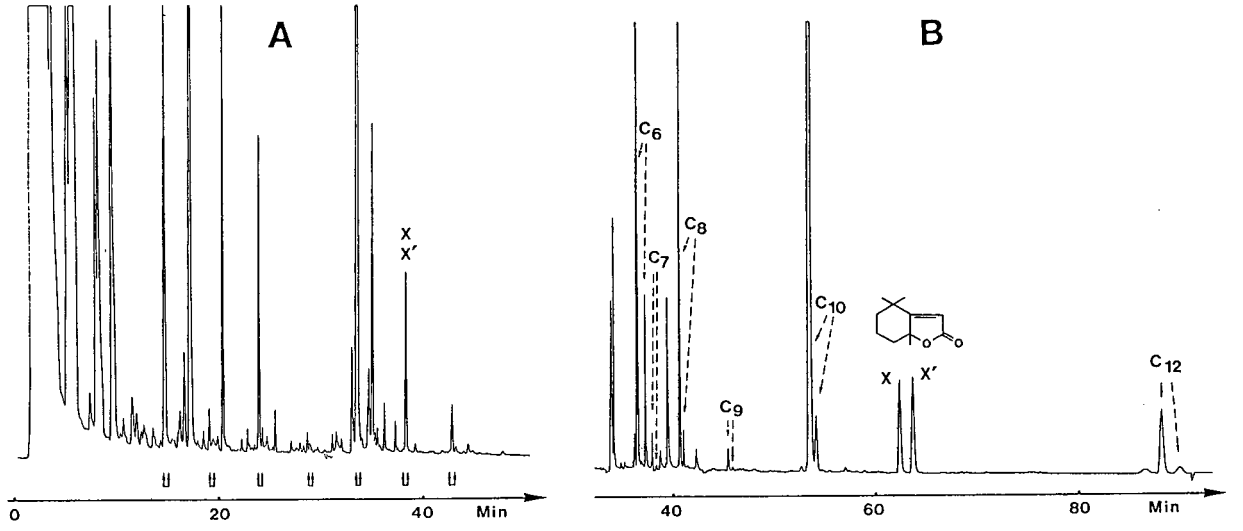


Fig. 7. (A) Raw apricot flavour extract, preseparated with DB-1701. (B) Chirality ealuation of γ-lactones from apricots, using modified β-cyclodextrin. From ref. 79.

al. [122] and their chromatographic behaviour interpreted by co-injection with optically pure references, as described by Palm *et al.* [86]. Using enantioselective MDGC and the column combination OV-1701–octakis(3-O-butyryl-2,6-di-O-pentyl)-γ-cyclodextrin, the simultaneous stereodifferentiation of all aroma relevant 4(5)-alkyl-substituted γ(δ)-lactones has been reported recently (Fig. 8) [92]. Only the less abundant lactone enantiomers (4*S*)-γ-heptalactone and (5*S*)-δ-hexalactone co-elute. If necessary, γ-heptalactone or δ-hexalactone can be analysed alternatively.

Peaches, apricots and greengages contain γ(δ)-lactones with characteristic enantiomeric ratios in favour of *R*-configured lactones. Whereas aroma-relevant amounts of γ-lactones from raspberries are not detectable, their δ-C_8, δ-C_{10}-lactones are optically pure *S*-enantiomers [92]. In cheddar cheese aroma the optical purity of (5*R*)-δ-lactones (C_{10}–C_{14}) increases with increasing side-chain length [114]. The enantioselective analysis of massoilactone (dec-2-en-5-olide), a coconut-typical δ-lactone of natural origin, has been reported by Bernreuther *et al.* [97] and Werkhoff *et al.* [114], revealing (−)-massoilactone of high optical purity (5*R*; >99% ee).

Alkan(alken)-2-yl acetates. Using enantioselective MDGC, the enantiomeric distribution of alkan(alken)-2-yl acetates from banana flavour was achieved. After simple acetylation their corresponding alcohols 2-pentanol (**1**), 2-hexanol (**2**), 2-heptanol (**3**) and (*Z*)-4-hepten-2-ol (**4**) are also exactly stereodifferentiated by this method. The main column chromatogram of these esters from a genuine banana flavour extract is shown in Fig. 9, indicating *S*-configured esters of high ee values [91].

The decreased optical purity of 2-pentanol and (*Z*)-4-hepten-2-ol from fully ripe bananas [91], also reported by Fröhlich *et al.* [123], is suggested to be caused by competitive enzyme activities during the latest stage of ripening but is not finally revealed as yet. Further studies, including enantio-MDGC and SIRA measurements, are in hand [124].

2-Alkylcarbonic acids (esters). 2-Alkylcarbonic acids have been separated into their enantiomers without any derivatization and their sequence of elution was assigned by co-injection with optically pure references [66]. Latest results on stereospecific flavour evaluation revealed characteristic sensory properties for all the enantiomers of 2-alkyl-branched acids, esters and corresponding alcohols. Tremendous differences between the mirror images of 2-methylbutanoic acid have been found. While

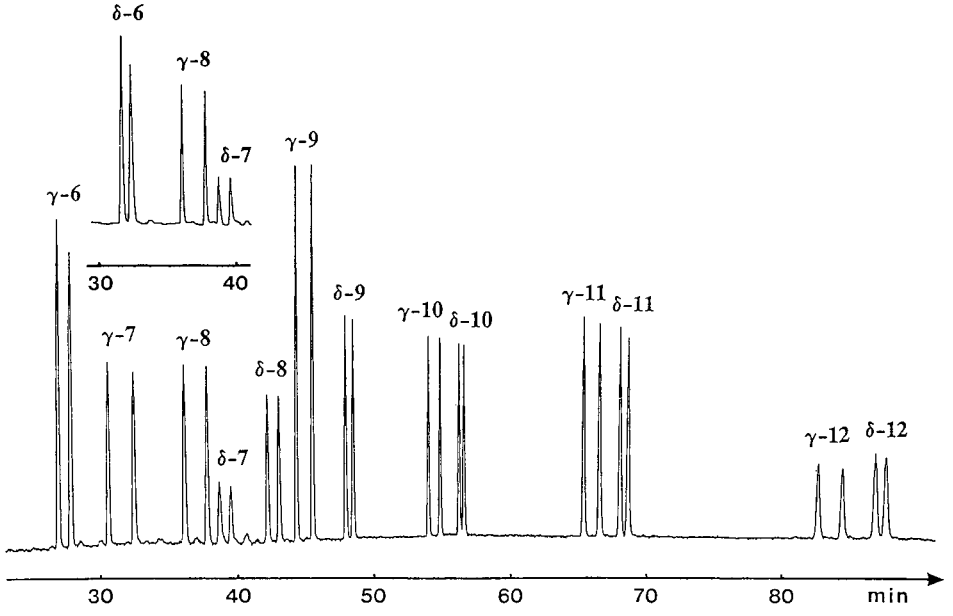


Fig. 8. Simultaneous stereodifferentiation of chiral γ(δ)-lactones using enantio-MDGC. From ref. 92.

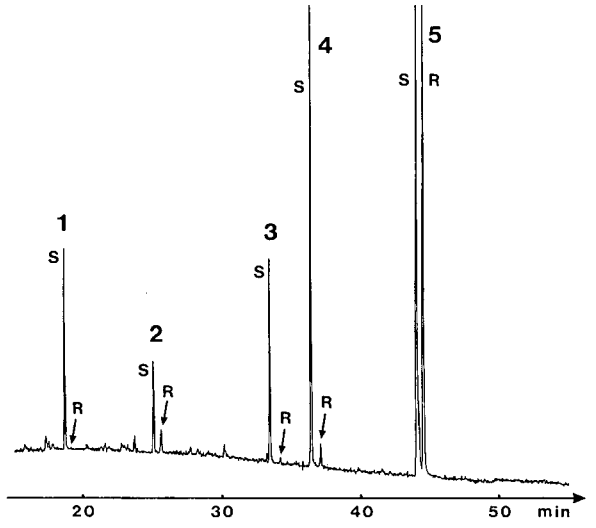


Fig. 9. *S*-Configured esters of (1) 2-pentanol, (2) 2-hexanol, (3) 2-heptanol and (4) (*Z*)-4-hepten-2-ol from a genuine banana flavour extract; (5) (*Z*)-4-hepten-2-yl-propionate, internal standard. From ref. 91.

the *R*-enantiomer exhibits a penetrating, cheesy-sweaty odour, the *S*-enantiomer emits a pleasant sweet and fine fruity note [125].

From the analytical point of view, it is worth noting the biogenetic pathway of 2-methylbutanoic acid starting from isoleucine [(2*S*)-amino-(3*S*)-methylpentanoic acid]. The *S*-configuration of the precursor is expected to remain, but also enzymatic racemization (by enolization of the intermediate 2-oxo-3-methylpentanoic acid) is known from the literature [126].

Appropriate analytical techniques without any racemization were developed and applied to apples and apple-containing foods. In all the investigated foods the (*S*)-enantiomer has been identified with high optical purity. Thus, the addition of nature-identical, synthetic 2-methylbutanoic acid racemate is easily detected [89,128].

The direct (but not baseline resolved) stereodifferentiation of ethyl 2-methylbutanoate, a well known impact flavour compound of the apple aroma, was reported simultaneously by Takeoka *et al.* [127] and Mosandl *et al.* [66]. Meanwhile further enantioselective procedures have been developed, indicating nearly optically pure (*S*)-ethyl 2-methylbutanoate as the unique antipode from natural flavours [89,128] and its impressive and pleasant apple note at extreme dilution has been recognized recently [125]. As the latest advance in this field the first simultaneous stereoanalysis of 2-methylbutanoic acid, its methyl (**1**) and ethyl (**2**) esters and its corresponding alcohol 2-methylbutanol (**3**) was realized, using selectivity-adjusted enantio-MDGC with perethylated β-cyclodextrin as the chiral stationary phase. All four chiral compounds were detected as optically pure *S*-enantiomers from apples. The application of this technique to a commercially available apple flavour concentrate reveals the adulteration by means of methyl (**1**) and ethyl ester (**2**) racemates (Fig. 10) [95].

Theaspirane stereoisomers. Theaspirane (2,6,10,10-tetramethyl-1-oxaspiro [4.5]dec-6-ene) has been found as a mixture of diastereomers in black tea [130], vanilla [131], raspberries [132,166], grapes and guavas [133,167] and the yellow passion fruit [134]. Using enantioselective analysis of synthetic theaspirane with permethylated β-cyclodextrin, both stereoisomers are resolved into racemic pairs of diastereomers [129]. Although the absolute configurations of theaspirane stereoisomers have not been elucidated so far, their optical activities were defined by polarimetric detection. It is interesting to note the preponderance of dextrorotatory diastereomers in osmanthus oil, whereas the levorotatory

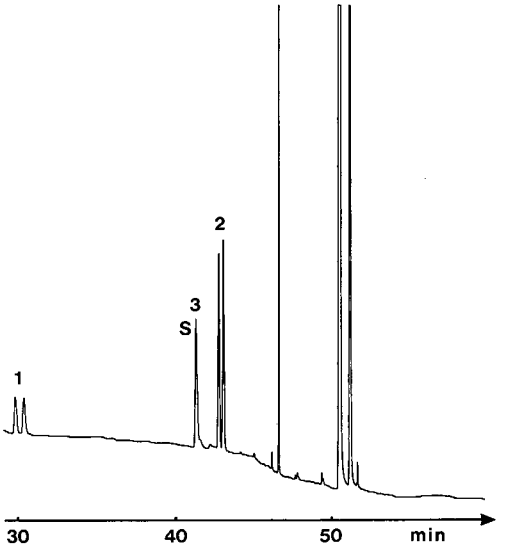


Fig. 10. Enantio-MDGC analysis of an apple flavour concentrate, adulterated by synthetic racemates of (1) methyl and (2) ethyl 2-methylbutanoate. From ref. 95.

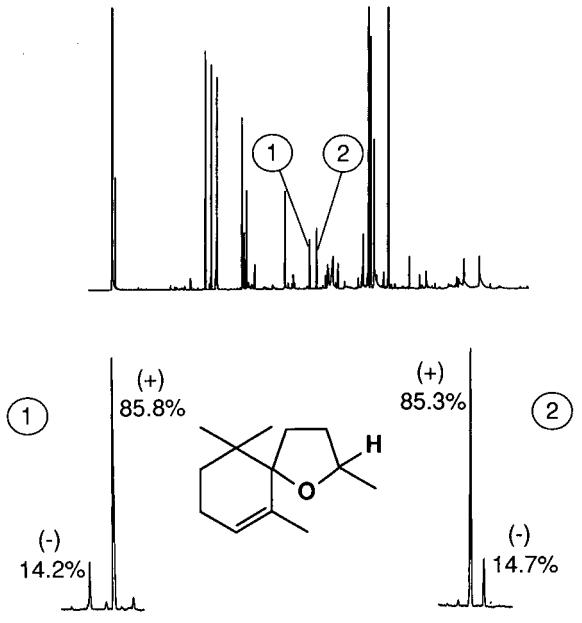


Fig. 11. Enantioselective analysis of theaspirane stereoisomers from *Osmanthus* oil. Top: achiral separation phase, 60 m × 0.32 mm I.D. DB-1 (0.25 μm); peak 1 separated on a chiral phase, see bottom left. Bottom right: the same for peak 2 from the achiral phase. Chiral separation phase: 25 m × 0.25 mm I.D. heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin. Bottom centre: structure of theaspirane. From ref. 114.

enantiomers predominate in authentic raspberry flavour (Fig. 11) [114].

Borneol, bornyl acetate, isoborneol. With respect to their natural origin, it seems to be reasonable to evaluate systematically chiral monoterpenes and derivatives. Borneol, bornyl acetate and isoborneol are well known and appreciated bicyclic monoterpenes that occur abundantly in nature, in particular in the essential oils of *Pinus* species.

The less expensive method of high-performance thin-layer chromatography (HPTLC) and subsequent extraction of the areas of interest has been successfully applied for the direct stereodifferentiation of borneol, bornyl acetate and isoborneol by one-dimensional cGC on permethylated β-cyclodextrin phase (Fig. 12). The method is recommended as a low-cost alternative and applicable with high accuracy and precision if definitive work-up conditions are observed, including (off-line) MS detection control [81].

The chromatographic behaviour of all the investigated 2-bornane derivatives was unambiguously assigned, referring to the enantiomers of camphor, which are preferably reduced by $LiAlH_4$ to the corresponding isoborneols (93%) of definite chirali-

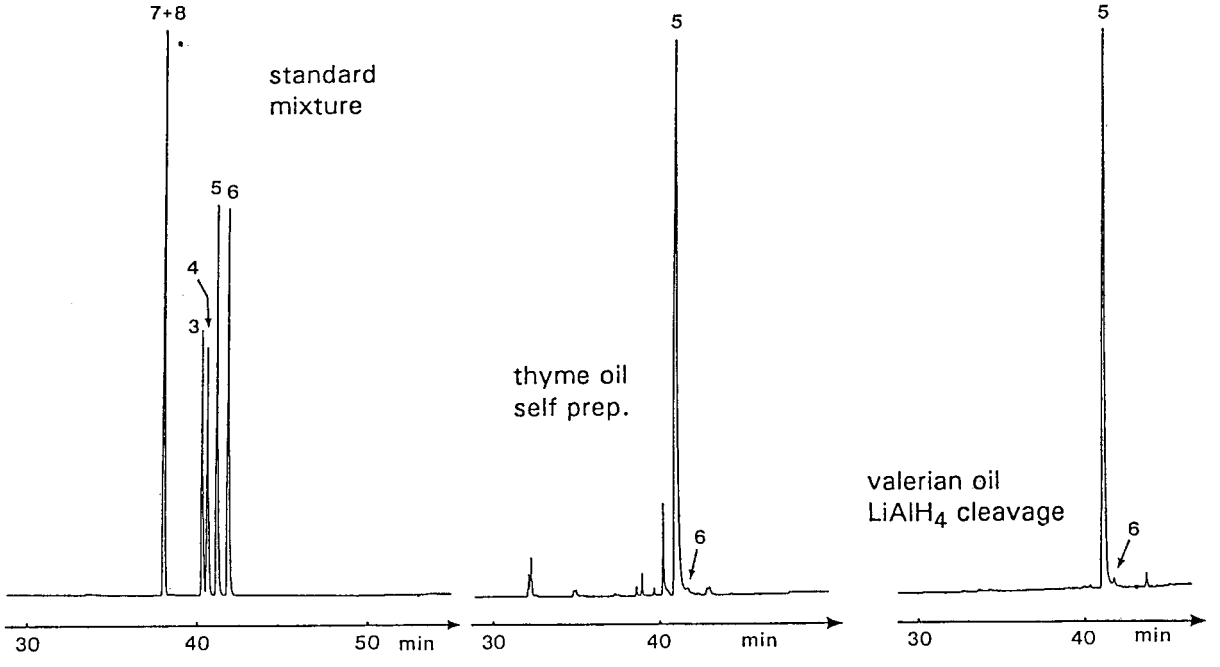


Fig. 12. Chirospecific analysis of (3,4) isoborneol, (5,6) borneol and (7,8) bornyl acetate [(−)-isomer first in each instance], with (−)-borneol (5) from thyme oil and valerian oil (after hydrolysis). From ref. 81.

ty (**3**,**4**). On the other hand, both bornyl acetates (**7**, **8**) are transferred to the corresponding borneols without any change in chirality by reductive ester cleavage.

Menthol, menthyl acetate, menthone, isomenthone. The essential oils of *Mentha* species are known to be valuable ingredients of pharmaceutical and cosmetic preparations. Menthone (**1**), isomenthone (**2**), menthol (**3**) and menthyl acetate (**4**), the most important *Mentha* oil constituents, are defined by their amounts as substantial parameters of *Mentha* oil quality. Racemic menthyl acetate was detected by Kreis *et al.* [80] as a frequently used adulterant of *Mentha* oils.

Two-dimensional GC in the direct enantiomer separation of **1–3** with $Ni(HFC)_2$ as the chiral main column was reported by Bicchi and Pisciotta [135]. Werkhoff *et al.* [114] isolated **1–4** from peppermint oils before stereoanalysis with permethylated β-cyclodextrin.

The simultaneous optical resolution of **1–4** has already been achieved using a combination of three different columns coated with modified cyclodextrins. All four enantiomeric pairs of **1–4** were directly stereoanalysed. Appropriate dilutions of mint and peppermint oils were analysed by a single chromatographic run and without any preseparation [145] (Fig. 13).

Linalyl acetate. Complexation GC with $Ni(HFC)_2$ as the chiral stationary phase [104] was found to be a convenient and effective method for stereoanalysing 1-octen-3-yl acetate in addition to linalyl acetate, the most important component of lavender oil [82, 135]. (*R*)-(−)-Linalyl acetate has to be considered as an indicator of the genuineness of lavender oil, as the optical purity of linalyl acetate is not influenced by the variety of lavender, and it is also independent from work-up and storage conditions [82].

Linalool. Linalool(3,7-dimethyl-1,6-octadien-3-ol) is widespread in plants and fruits and is one of the most frequently used compounds in flowery fragrance compositions. As linalool is an important intermediate in vitamin E synthesis, several large-scale processes for its production have been developed [136]. The stereo- and origin-specific analysis of linalool is of fundamental interest.

Perpentylated β-cyclodextrin [67,137] and the perethylated derivative [93] have been synthesized and applied successfully in the enantioselective MDGC of linalool from essential oils and fruits

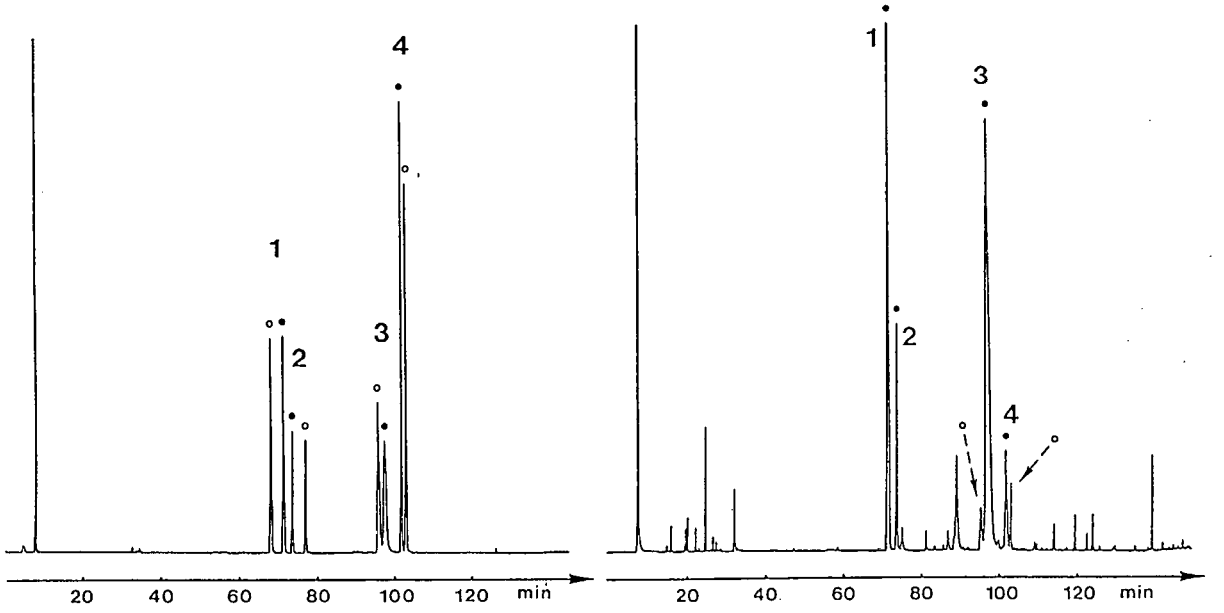


Fig. 13. Simultaneous optical resolution of (1) menthone, (2) isomenthone, (3) menthol and (4) menthyl acetate. Left, standard mixture; right, adulterated mint oil. (●) Genuine constituents of mentha oils; (○) enantiomers not detectable in mentha oils. From ref. 145.

[94,114,138]. However, referring to a proposal of Rapp and Mandery [139], the reversible hydration of linalool to geraniol or nerol in acidic media should result in an equilibration of linalool's optical activity to yield racemates. Indeed, linalool, α-terpineol and linalool oxides (fur.) were detected as racemates from a 3-year-old bottle of matured white wine [93]. On the other hand, comparing the enantiomer distributions of linalyl acetate and linalool from lavender species, high optical purities in favour of the (*R*)-(−)-enantiomers were found in laboratory-prepared diethyl ether extracts and in officinal steam distillates of lavender. However, in the case of prolonged times of hydrodistillation (≥1 h), increasing amounts of (*S*)-(+)-linalool (5–15%) are detectable. The genuine main component of lavender oil, (*R*)-(−)-linalyl acetate, is hydrolysed in considerable amounts during hydrodistillation, but nevertheless the absolute configuration of the ester remains unchanged [140,168]. Hence, enantioselective analysis of linalool, α-terpineol and linalool oxides (fur.) will not conclusively reflect their genuine enantiomeric ratios in any case, as the chiral stability of linalool (and its cyclic derivatives) obviously depends on the stage of fruit ripeness, pH value of plant materials and technological influences.

1-Octen-3-ol. 1-Octen-3-ol is one of the character-impact flavour compounds of mushrooms [141] and is formed by enzymic oxidative breakdown of linoleic acid [142]. (*R*)-(−)-1-octen-3-ol is responsible for the fruity soft odour, in accordance with the genuine mushroom note [38]. High ee values in favour of the (*R*)-(−)-antipode have been identified in mushrooms via diastereomeric esters with Mosher's acid [42], (*S*)-O-acetyllactic acid [143] and by enantiomeric resolution of 1-octen-3-yl-acetate [101].

As shown in Fig. 14, the direct and simultaneous stereodifferentiation of 1-octen-3-ol, 3-octanol and linalool is available as a valuable tool in the enantioselective analysis of many flavours and fragrances [110].

Nerolidol. Nerolidol, a sesquiterpenoic alcohol, analogous in structure to linalool, is a valuable base note in perfumery, owing to its long-lasting and moderate floral odour. Recently nerolidol was separated into its four stereoisomers via MPLC of the diastereomeric (1*S*,4*R*)-camphanoates and subsequent ester hydrolysis. Distinct sensory properties of all stereoisomers and a chirospecific cGC method to resolve the enantiomeric pairs of (*Z*)- and (*E*)-nerolidol were described (Fig. 15) [144].

8-Mercapto-p-menthan-3-one. Owing to their characteristic minty–fruity notes, reminiscent of blackcurrants, buchu leaf oils are well appreciated in the composition of flavours and fragrances. 8-Mercapto-*p*-menthan-3-one and its thiolacetate have been described by Sundt *et al.* [158] and independently by Lamparsky and Schudel [159] as impact flavour compounds of cassis flavours. They have not so far been identified in blackcurrant. All four stereoisomers of 8-mercapto-*p*-menthan-3-one exhibit distinct and characteristic sensory impressions and their first chirospecific analysis was described by Köpke and Mosandl [29] (Fig. 16).

Rose oxides. Rose and geranium oils are precious natural products in fine perfumery. The diastereomers of rose oxides are known to be characteristic chiral constituents and attributed to the distinct bloomy-green top notes of rose and geranium oils. The first enantiomeric separation of both *cis* and *trans* rose oxides using complexation GC was published by Schurig [160]. Werkhoff *et al.* [114] reported on the chirospecific analysis of *cis* and *trans* rose oxides from geranium oil after preseparation by preparative cGC and alternative chirospecific analysis of *cis–trans* diastereomers, using two different modified γ-cyclodextrins.

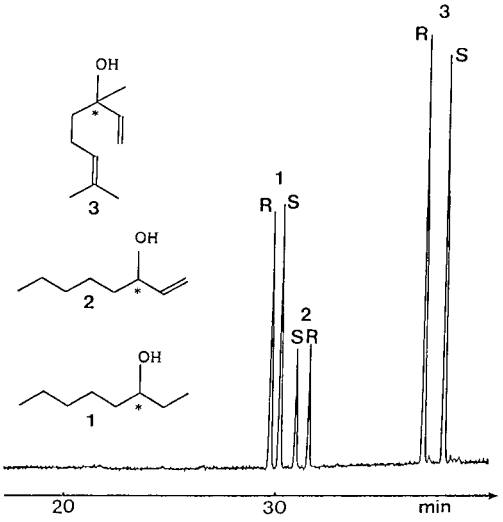


Fig. 14. Chirospecific differentiation of (1) octanol, (2) 1-octen-3-ol and (3) linalool. From ref. 110.

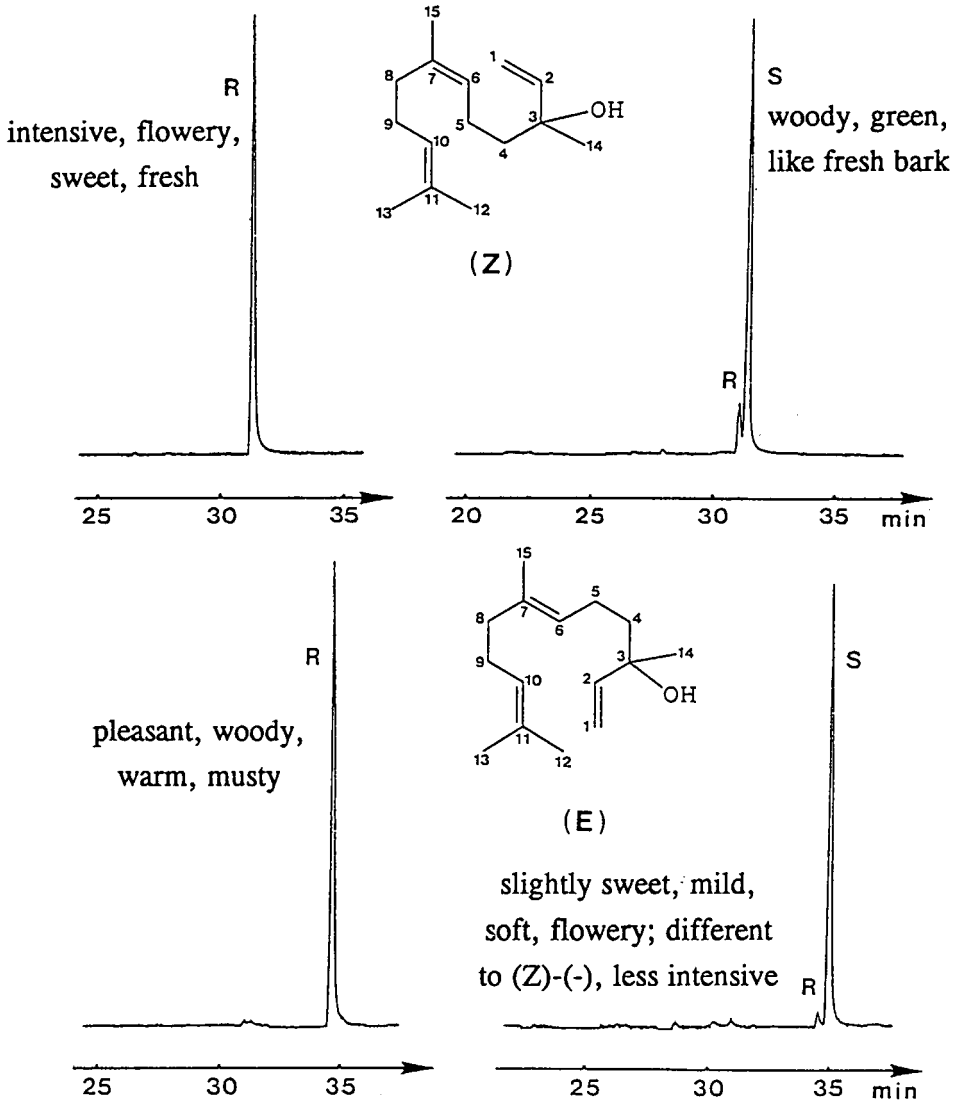


Fig. 15. Sensory description of nerolidol stereoisomers and enantiomeric purity control using heptakis(2,3,6-tri-O-methylhydroxypropyl)-β-cyclodextrin. From ref. 144.

2,3-Di-O-acylated cyclodextrin derivatives were introduced as a new type of chiral stationary phases in cGC [109]. Heptakis(2,3-di-O-acetyl-6-O-*tert.*-butyldimethylsilyl)-β-cyclodextrin is reported as a versatile stationary phase for the simultaneous stereodifferentiation of a wide range of chiral volatiles with different functionalities. The simultaneous chiral resolution of *cis–trans* rose oxides, citronellol and linalool, the most important chiral compounds of rose and geranium oils, including the complete interpretation of the chromatographic behaviour of all stereoisomers investigated, is the latest result in this field, reported by Dietrich *et al.* [110] (Fig. 17). Although there are tremendous difficulties in obtaining genuine rose or geranium oils, their authenticity control by enantio-MDGC has been achieved [140,168].

3-(2H)-Furanones. Cyclopentenolones with a

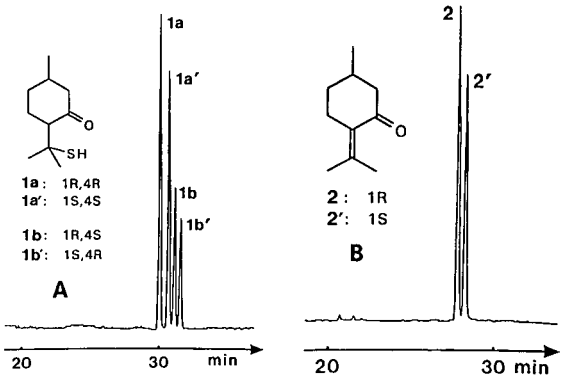


Fig. 16. Chirospecific analysis of (A) 8-mercapto-*p*-menthan-3-one and (B) pulegone on permethylated β-cyclodextrin phase. From ref. 29.

planar vicinal enol–oxo configuration are known to be powerful aroma-active substances with distinct caramel notes. By methylation of the enolic function, this flavour impression is changed drastically to a sweet, mildew and mouldy odour in the case of 2,5-dimethyl-4-methoxy-3-(2*H*)-furanone (**2**). This so-called "mesifurane" and also "pineapple ketone" (**1**) were stereodifferentiated with modified cyclodextrin [154]. Although (**1**) and (**2**) can be stereoanalysed without any racemization, both compounds were detected in strawberries, pineapples, grapes and wines as racemates (Fig. 18).

Obviously optically active furanones are equilibrated by keto–enol tautomerism in acidic media. Thus, enantioselective analysis cannot be applied to assign the origin of chiral 3-(2*H*)-furanones [108]. This can only be expected by stable isotope measurements [151].

4.2. Stable isotope ratio mass spectrometry (IRMS)

4.2.1. cGC–IRMS

The elucidation of stable isotope distributions is highly desirable with respect to fundamental studies in biochemistry, in nutrition and drug research and in the origin assignment and authenticity control of flavours and fragrances. cGC coupled on-line via a combustion interface with isotope ratio mass spectrometry (IRMS) has been realized successfully in the case of $\delta^{13}C$ determinations [146]. The substances eluting from the cGC column are converted into carbon dioxide in a combustion oven and then directly analysed in the isotope mass spectrometer, adjusted for the simultaneous recording of masses 44 ($^{12}C^{16}O_2$), 45 ($^{13}C^{16}O_2$; $^{12}C^{16}O^{17}O$) and 46 ($^{12}C^{16}O^{18}O$) in the nmol range and with high precision $\leqslant 0.3‰$).

Owing to the possible ion–molecule reaction between H_2O and CO_2, HCO_2^+ ions may be generated, simulating the existence of $^{13}CO_2$. Hence the

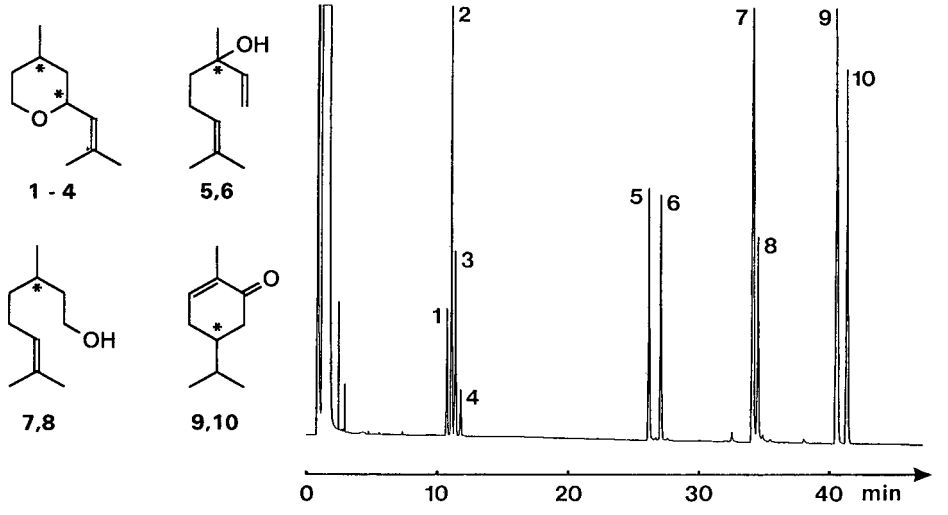


Fig. 17. Simultaneous stereodifferentiation of chiral rose oil compounds. Rose oxides: *cis* isomers (2*R*,4*S*)-(+)-**1**, (2*S*,4*R*)-(−)-**2**; *trans* isomers (2*R*,4*R*)-(−)-**3**, (2*S*,4*S*)-(+)-**4**. Linalool: (*R*)-(−)-**5**, (*S*)-(+)-**6**. Citronellol: (*S*)-(−)-**7**, (*R*)-(+)-**8**. Carvone: (*R*)-(−)-**9**, (*S*)-(+)-**10**. From ref. 110.

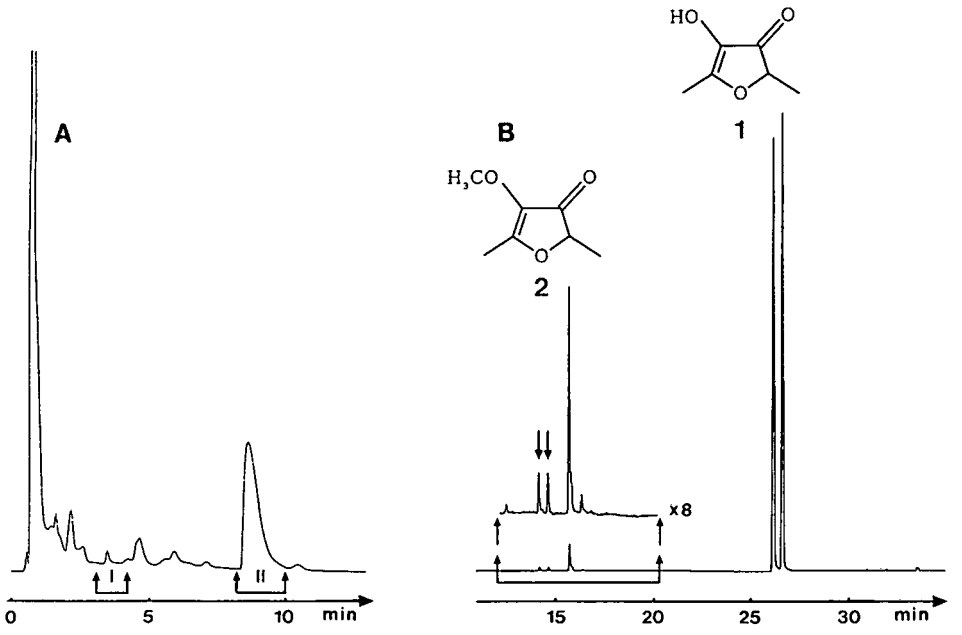


Fig. 18. (A) HPLC of a raw pineapple flavour extract. (B) Enantioselective analysis of 3-(2*H*)-furanones (1 and 2) from HPLC eluates I and II. From ref. 108.

combustion product, H_2O, must be removed just behind the furnace either by a capillary-shaped phase separator, made of water-permeable membrane materials [115,147], or by a capillary cryogenic trap [148].

In comparison with classical IRMS methods, the instrumental configuration of cGC–IRMS combines the precision of IRMS with the high purification effect of cGC separation, with large savings on laborious sample clean-up procedures. Isotope ratios are calculated relative to standard values, commonly defined as δ-values, where the δ-value is the relative difference in the isotope ratio of the sample (R_{sa}) to that of the standard (R_{st}), given in parts per thousand [146]:

$$\delta = \left(\frac{R_{sa}}{R_{st}} - 1\right) \cdot 1000\,(‰)$$

The isotope ratio traces of the GC peaks exhibit a typical S-shape, caused by a vapour pressure isotope effect, eluting the heavier isotopic species of a compound more rapidly from the cGC column than the light species. The actual ratio is computed from the ratio of the areas of the two isotopic peaks. Hence, care must be taken to integrate across the full width of the chromatographic peaks [147]. Of course, reliable results on isotope ratios from cGC–IRMS experiments can only be expected from real high-resolution cGC. Also, accurate sample clean-up procedures without any isotope fractionation must be guaranteed [120,148,149].

4.2.2. *Applications*

γ-Lactones. Alternative investigations of γ-decalactone from various sources were reported by Bernreuther *et al.* [96], confirming high ee values in favour of the 4*R*-enantiomer [76–79], whereas $\delta^{13}C_{PDB}$ values differ in relation to their origin (Table 2). The most striking deviations in $\delta^{13}C_{PDB}$ values were detected in the group of stone fruits. With the present state of knowledge final explanations cannot be given.

Blends of γ-decalactone from different origins were analysed in model experiments, using enantioselective cGC, coupled on-line with IRMS [enantio-SIRA(IRMS)], to demonstrate the advantages of enantio-SIRA, although some decrease in sensitivity has to be taken into account in comparison with

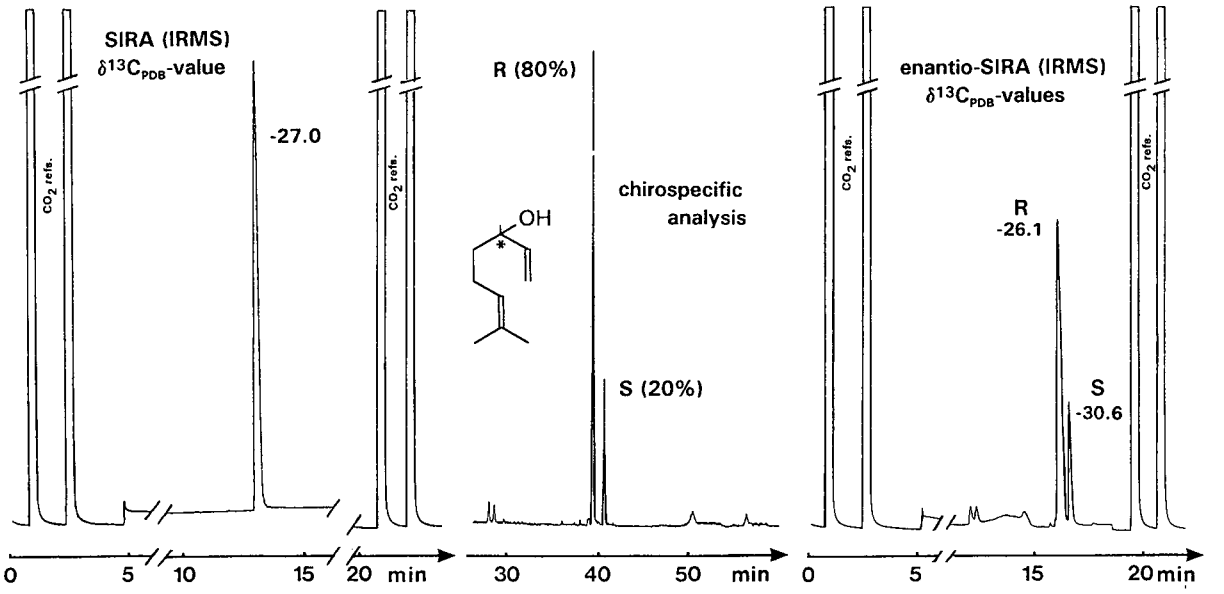


Fig. 19. cGC–IRMS, chirospecific analysis and enantio-SIRA measurements of linalool from a commercially available spike oil. $\delta^{13}C_{PDB}$ values (‰): linalool −27.0 (±0.2); *R*-enantiomer −26.1 (±0.2); *S*-enantiomer −30.6 (±0.6). From ref. 161.

cGC–IRMS using non-chiral stationary phases [150]. Enantio-IRMS detects enantiomers of the same source with identical $\delta^{13}C$ levels. Enantio-IRMS also offers a direct method to detect conclusively a blend of optically pure chiral flavour compound with synthetic racemate: a fruit-specific enantiomeric ratio may be imitated but is not yet detectable neither by chirospecific analysis nor by IRMS measurements. However, in the case of enantio-IRMS a simulated fruit-specific enantiomeric distribution is proved by different $\delta^{13}C$ levels of the detected enantiomers.

Furthermore, enantiomers with identical $\delta^{13}C$ levels are expected from genuine compounds, even if the structural features of chiral molecules to be analysed may be sensitive to (partial) racemization. On the other hand, it seems improbable that the same biological organism pursues different biochemical pathways to synthesize precursors of mirror images. Hence, enantio-SIRA measurements indicating significantly different $\delta^{13}C_{PDB}$ values of the investigated enantiomers should be explained as a blended compound of different origin (Fig. 19).

As outlined in Fig. 19, cGC–IRMS detects the $\delta^{13}C_{PDB}$ value of linalool from a commercially available spike oil in the presumed natural range of

TABLE 2

^{13}C CONTENT OF γ-DECALACTONE ORIGINATING FROM VARIOUS SOURCES [96]

Except for the value given for apricot, which showed a variation of ±2.0‰ due to interfering peaks, for the other HRGC–IRMS determinations variations of ±0.5‰ were found. For the conventional measurements ±0.2‰ was determined.

Origin	$\delta^{13}C_{PDB}$ (‰)	
	HRGC–IRMS	Conventional
Natural		
Strawberry		
Spadeka	−28.2	
Bogota	−28.9	
Bel Ruby	−30.5	
Peach		
Italy	−40.9	
Germany	−38.5	
Plum (Mirabelle)	−39.6	
Apricot	−38.0	
Microbial (A)	−31.2	−30.8
Microbial (B)	−30.3	−30.7
Synthetic		
Aldrich	−26.9	−27.3
Roth	−24.4	
Takasago	−26.0	

(−26.0 to −28.2‰), whereas chirospecific analysis and enantio-SIRA measurements will allow much more conclusions with respect to the authenticity control of this chiral compound. Systematic investigations are in hand to confirm these results [161].

Origin-specific analysis of (E)-α(β)-ionone. (*E*)-α-Ionone from raspberries and many other natural sources occurs as the optically pure (*R*)-(+)-enantiomer [151,152]. Thus, nature-identical (*E*)-α-ionone racemate from *Boronia* absolue and optically pure (*R*)-(+)-(*E*)-α-ionone from a genuine oil of *Osmanthus* are detected unambiguously [114] (Fig. 20).

The sensory quality of (*E*)-α-ionone racemates is not equivalent to the *R*-configured (*E*)-α-ionone [152], as concluded from the sensory characteristics of (*E*)-α-ionone enantiomers [151]. Using stable isotope mass spectrometry, (*E*)-α- and (*E*)-β-ionone from raspberries are well differentiated from their synthetic analogues and from a commercially available product, which was declared to be of natural origin [152] (Table 3).

New clean-up procedures. Nitz *et al.* [148] reported the first application of multi-dimensional GC coupled on-line to isotope mass spectrometry (MDGC–IRMS) (Fig. 21). This highly sophisticated and elegant method will be of considerable interest in the field of flavour analysis. However, major restrictions must also be taken into account if δ-values from sub-nanogram samples are to be evaluated from complex mixtures in the presence of extreme amounts of other substances. With respect to the minimum IRMS sensitivity level (about 1 nmol of substance to be analysed), direct MDGC–IRMS might be insufficient, in spite of a high sample loading provided by a thick-film precolumn capillary.

Preparative high-resolution segment chromatography (PHSC) has been applied successfully as an alternative to MDGC in sample clean-up for the cGC–IRMS analysis of orange oils [149].

Fig. 22 compares a genuine orange oil with the same oil after separating (*R*)-(+)-limonene (L) by

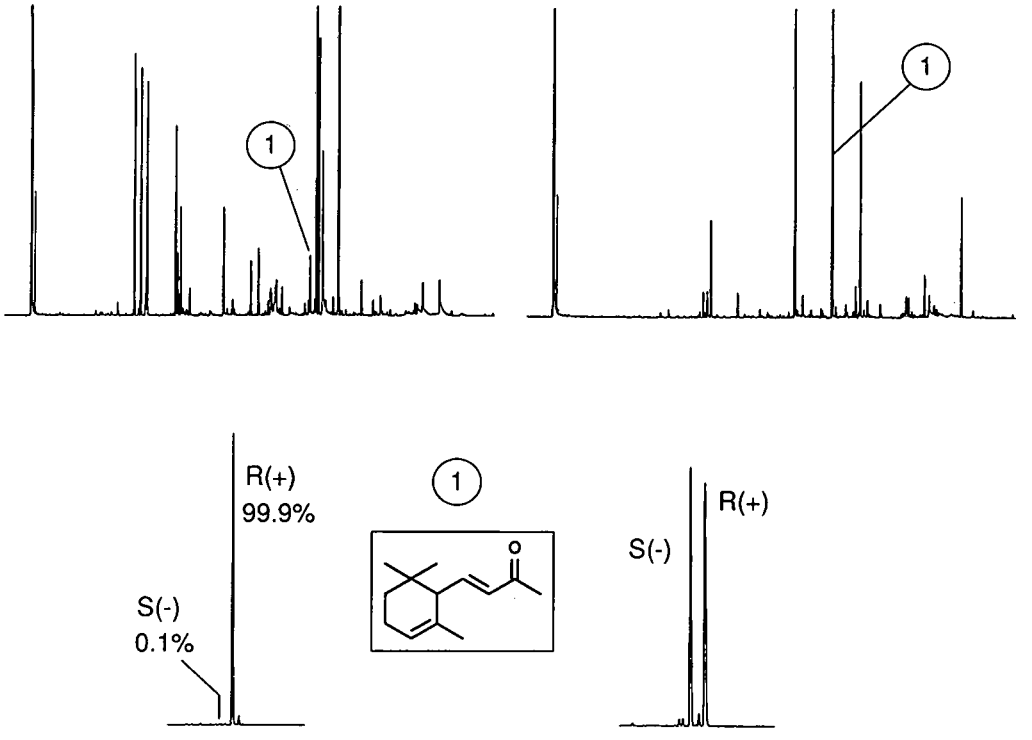


Fig. 20. Chirospecific analysis of (*E*)-α-ionone of *Osmanthus* oil (left) and *Boronia* absolue (right). Top: cGC analysis with achiral column, 60 m × 0.32 mm I.D. DB1 (0.25 μm). Bottom left: separation of naturally occurring enantiomers in *Osmanthus* oil. Bottom right: separation of enantiomers of commercially available *Boronia* absolue. Chiral column, 25 m × 0.25 mm I.D. heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin. Bottom centre: structure of (*E*)-α-ionone. From ref. 114.

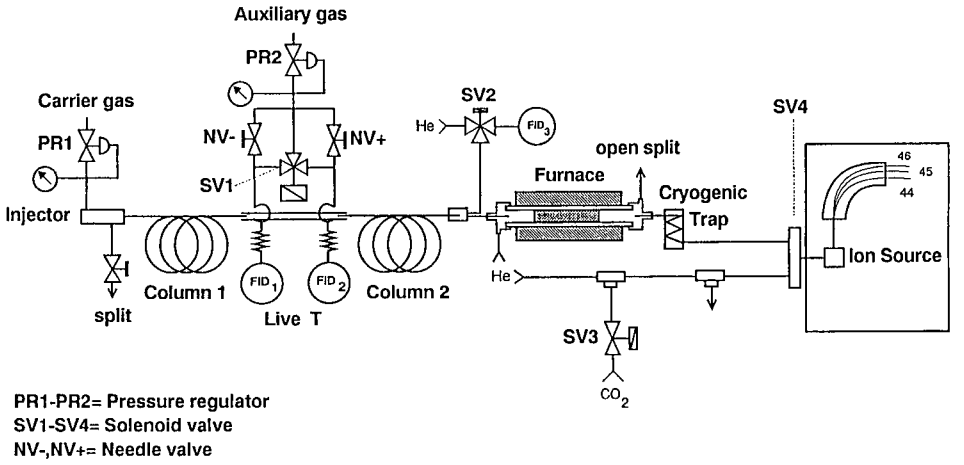


Fig. 21. Schematic diagram of MDGC–IRMS. From ref. 148.

PHSC on silica gel and ready for one-dimensional cGC–IRMS analysis. Every step during sample clean-up was achieved without any isotope fractionation. PHSC has proved to be an inexpensive preseparation and simple concentration step in the cGC–IRMS analysis of fatty aldehydes, in particular of octanal and decanal, two minor compounds which contribute substantially to the sensory quality of cold-pressed orange oils.

The results, shown in Table 4, are remarkable, in particular for decanal, one of the main aldehydes in orange oil. The $\delta^{13}C$ values vary in a small range (about 1‰), independent of variety, provenance and growing conditions [149].

4.3. *Trends and perspectives*

cGC coupled on-line with IRMS is one of the latest developments, useful in the origin control of flavour and fragrances. While genuine flavour compounds from C_4 plants differ significantly from those of C_3 plants, partial overlapping ranges of $\delta^{13}C$ values of substances from C_3 plants with those prepared from fossil sources and from microbial origin have to be accepted. Further, the incorporation of the isotope abundances, especially $\delta^{13}C$ and $\delta^{2}H$, has enhanced the capability to determine the authenticity of cassia and bitter almond essential oils [54,154].

Three principles in the future progress of stable

TABLE 3

$^{13}C_{PDB}$ VALUES OF (*E*)-α(β)-IONONE FROM DIFFERENT ORIGINS [152]

Origin	(*E*)-α-Ionone			(*E*)-β-ionone
	(*R*)-(+)- (%)	(*S*)-(−)- (%)	$\delta^{13}C_{PDB}$ (*s*)[a]	$\delta^{13}C_{PDB}$ (*s*)[a]
Synthetic A	50	50	−24.33 (0.16)	n.d.[b]
Synthetic B	50	50	−26.69 (0.10)	n.d.
Synthetic C	n.d.	n.d.	n.d.	−28.63 (0.22)
Synthetic D	50	50	−27.05 (0.15)	−25.59 (0.09)
Raspberry (fruit)	>99.9	<0.1	−32.86 (0.59)	−33.41 (0.37)
Raspberry (mash)	>99.0	<0.1	−33.43 (0.38)	−31.41 (0.38)
Fermentative	50	50	−9.12 (0.06)	−8.57 (0.16)

[a] Standard deviations (*s*) are given in parentheses ($n = 5$).
[b] n.d. = Not detectable.

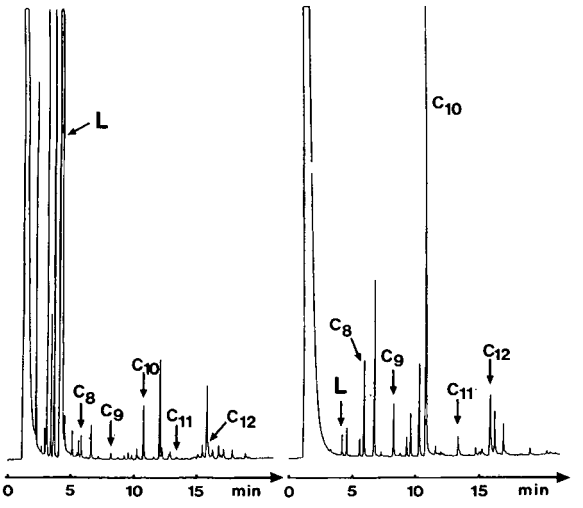


Fig. 22. cGC analysis of a genuine orange peel oil, (left) laboratory prepared and (right) after separating (*R*)-limonene using PHSC. L = limonene (L); C_8–C_{12} = aldehydes. From ref. 149.

isotope analysis were defined by Schmidt *et al.* [155]: multi-compound analysis, using cGC–IRMS (Section 4.2.1); multi-element analysis, replacing the combustion unit by a pyrolytic converter to produce CO, N_2 and H_2 from organic material and analysis by on-line IRMS; and automated positional isotope analysis from the combination of a controlled pyrolysis with IRMS analysis.

In particular, on-line hydrogen, nitrogen and oxygen stable isotope analysis in organic compounds and water by interfacing cGC with IRMS via a catalytic Pt–Rh capillary reactor is the latest progress in cGC–IRMS and may open up exciting perspectives in many biosciences [155]. In addition, chirality evaluation as an indicator of the naturalness of flavour and fragrance compounds has advanced tremendously.

While the direct analysis of single chiral compounds and their homologues [76–79] was reported in the first stage of enantioselective flavour analysis, simultaneous stereodifferentiation of chiral compounds with different functionalities has subsequently been achieved [92,95]. Developing tailor-made chiral columns for the direct and simultaneous enantioselective analysis of most chiral impact compounds of rose oil or lavender oil is the latest advance in this field of research [110,140] and indeed remains a challenge.

5. CONCLUSIONS

Aroma extract dilution analysis (AEDA) and stable isotope dilution assay (SIDA) have proved to be valuable tools in the quality assessment of flavours and fragrances. Concerning the genuineness of natural compounds, the importance of comparative stable isotope ratio mass spectrometry (IRMS) and chirality evaluation is increasing. Promising developments in both fields of research

TABLE 4

$^{13}C_{PDB}$ VALUES OF C_8–C_{12} ALDEHYDES FROM ORANGE PEEL OILS OF DIFFERENT ORIGINS [149]

Origin	$\sigma^{13}C_{PDB}$ (*s*)[a]			
	Octanal	Nonanal	Decanal	Dodecanal
Navel A[b]	−27.40 (0.31)	−28.31 (0.55)	−26.10 (0.36)	−27.13 (0.45)
Navel B[b]	−29.15 (0.28)	−28.23 (0.39)	−25.89 (0.16)	n.s.[d]
Navel C[b]	−28.25 (0.21)	−28.07 (0.23)	−26.11 (0.15)	−28.15 (0.17)
Valencia[b]	−28.02 (0.06)	−27.51 (0.25)	−25.75 (0.10)	n.s.
Siracusa[b]	−26.72 (0.24)	−26.80 (0.30)	−25.33 (0.16)	−27.10 (0.31)
Italy[c]	−27.67 (0.09)	−27.33 (0.25)	−26.60 (0.13)	n.s.
Florida[c]	−28.42 (0.23)	−28.60 (0.24)	−26.93 (0.19)	−27.60 (0.34)
California[c]	−27.96 (0.17)	−27.71 (0.08)	−26.34 (0.35)	−27.22 (0.40)

[a] Standard deviations (*s*) are given in parentheses ($n = 5$).
[b] Laboratory-prepared orange peel oils.
[c] Commercially available orange peel oils.
[d] No quantitative separation by GC as yet.

will strongly accelerate insights into the origin of flavours and fragrances. Continuous advances in analytical origin assignment will be adopted for quality assurance in the flavour industry and also should reflect legal regulations as a consequence.

6. ABBREVIATIONS

A	aroma value
a	odour threshold
AEDA	aroma extract dilution analysis
c	concentration
cR_s	chiral resolution
cGC	capillary gas chromatography
cGC–IRMS	capillary gas chromatography coupled on-line with isotope ratio mass spectrometry
CHARM	combined hedonic response measurements
CI	chemical ionization
ee	enantiomeric excess
EI	electron impact ionization
enantio-MDGC	enantioselective MDGC
enantio-SIRA(IRMS)	enantioselective cGC coupled on-line with SIRA(IRMS)
FD factor	flavour dilution factor
FID	flame ionization detection
FT-IR	Fourier transform infrared spectroscopy
fur.	furanoid
GC	gas chromatography
GC–O	gas chromatography–olfactometry
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
IRMS	isotope ratio mass spectrometry
Lipodex D	glass capillary column, coated with heptakis(2,6-di-O-pentyl-3-O-acetyl)-β-cyclodextrin
MDGC	multi-dimensional gas chromatography
MDGC–IRMS	multi-dimensional gas chromatography coupled on-line with isotope ratio mass spectrometry
MIM	multiple ion monitoring
MS	mass spectrometry
OAV	odour activity value
PHSC	preparative high-resolution segment chromatography
s	standard deviation
SIDA	stable isotope dilution assay
SIM	single ion monitoring
SIRA	stable isotope ratio analysis
SWC	selected wavelength chromatogram
Δt	absolute difference in retention time of two peaks
w_h	peak width at half-height
w_b	peak width at base (4σ)
σ	peak variance

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Review

Chromatography of tea constituents

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ABSTRACT

Modern chromatographic techniques such as high-performance liquid chromatography are currently the most helpful approach to the routine analysis of and research of non-volatile tea constituents. Using these techniques some errors in the more classical analytical techniques could be detected. Unfortunately, some of these methods of analysis are still in widespread use, even as official methods. However, knowledge of especially the polyphenols in tea is still lacking, and for many of the minor polyphenols no chromatographic methods for the determination exist.

CONTENTS

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1. INTRODUCTION

Tea is one of the most popular beverages in the world. In China and Japan usually the green (non-fermented) tea is consumed, whereas in India and most European countries black (fermented) tea is preferred. The major tea-producing countries are India, China, Japan, Sri Lanka, Indonesia and Central African countries. Tea production was about 2.2 million tons in 1984 [1]. Until now the quality assessment of tea has been performed worldwide by professional tea tasters [2]. For a long time, attempts have been made to correlate their findings with contents of chemical constituents.

The objectives of chemical analysis of tea cover at least three different areas: (a) to find a constituent or a group of tea constituents which are a measure of tea quality; (b) to optimize tea technology; and (c) to correlate the health effects of tea with certain tea constituents.

As regards the non-volatile constituents, in the past in most instances groups of tea constituents (*e.g.*, TFs, TRs) have been determined as a sum parameter using non-chromatographic methods. Chromatographic methods (PC, TLC) were developed in the 1950s and 1960s. Since 1976, many HPLC methods have been developed for tea alkaloids and pigments.

In this review we compare the more recently developed chromatographic methods (*e.g.*, HPLC) for the determination of natural tea constituents and more classical approaches. The determination of, *e.g.*, pesticide residues is not covered. More general information about tea (horticulture, technology, chemical composition) can be obtained, *e.g.*, from reviews by Bokuchava and Skobeleva [3,4] and Wickremasinghe [5] and from monographs [6–8].

2. ANALYSIS OF TEA CONSTITUENTS

2.1. Alkaloids

The different health effects of caffeine have been compiled recently by James [9]. Another recent paper deals with the metabolism of methylxanthines in tea and other beverages, including analytical aspects [10]. Black tea contains normally 1.5–4% of caffeine, 0.2–0.4% of theobromine and about 0.02% of theophylline.

2.1.1. Non-chromatographic methods

The most widely used non-chromatographic method is a modified Levine procedure [11]. The caffeine is extracted from ammonia-containing tea solutions, clean-up is performed on two columns (acidic, alkaline) and caffeine is eluted from the column using chloroform. Absorbance is measured at three different wavelengths, which has to be done to subtract interferences from other compounds. This procedure leads to reproducible results.

Gravimetric determination after extraction of the brews [$Ba(OH)_2$ added] with chloroform has also been used [12]. A similar procedure, using $Pb(OAc)_2$, KOH and $KMnO_4$ solutions, treatment with H_2SO_4 and iodine, followed by titration with $Na_2S_2O_3$, was reported [13].

2.1.2. HPLC methods

Numerous HPLC methods for the detection of tea alkaloids have been published, but in most countries the official methods are still non-chromatographic.

Clean-up for HPLC analysis is performed, *e.g.*, using heavy magnesium oxide [14] in a modified Kjeldahl apparatus [15] or in a shaking water-bath for 20 min [16]. In an international interlaboratory test an HPLC system with RP-18 phases and methanol–water (*ca.* 40:60, depending on the column used) was recommended. This procedure is simple and rapid to perform and leads to reproducible results, even for decaffeinated teas. It has been tested successfully in international and national ring tests [16].

Another clean-up procedure prior to HPLC is the use of a Sep-Pak C_{18} cartridge and a PVPP column for aqueous extracts of green tea as carried out by Kuwano and Mitamura [17]. The eluate was analysed by HPLC, using an RP-18 (Nova-Pak C_{18}) column and acetonitrile–water (20:80) as the mobile phase and monitoring at 254 nm.

Alternatively, if theobromine also should be detected, a clean-up by means of ion exchange (Dowex 1-X4) and elution with methanolic phosphate buffers has been proposed and is currently being tested as a German standard method [18,19]; the HPLC elution systems can be water–methanol or water–acetonitrile.

Kunugi *et al.* [20] used an Extrelut column clean-up with 8 *M* H_2SO_4 and 2 *M* NaOH (elution with

dichloromethane) and chromatography on a LiChrosorb Si 60 column. Water-saturated dichloromethane–ethanol (97:3) was used as a solvent. Some workers have described a clean-up by column chromatography using Al_2O_3 or MgO phases to remove polyphenols prior to RP-HPLC [21,22]. In most papers RP-HPLC separations are described. It is also possible to use normal-phase HPLC on silica layers [eluent, dichloromethane–methanol, (90:10)] as described, *e.g.*, by Vergnes and Alary [23].

A comparison between a spectrophotometric method and HPLC after chloroform extraction and water extraction–PVPP clean-up has been made [24]. Unfortunately, it was impossible to compare the results of the different methods owing to the different extraction conditions used (water and chloroform extractant, respectively).

HPLC (10-μm Bondapak C_{18} column with acetonitrile–2% acetic acid as eluent) and GLC (column: OV-17 on Chromosorb W HP; temperature programming) determinations have been compared [25]. Clean-up was done by liquid–liquid extraction (chloroform containing 5% isopropanol from alkaline brews). HPLC gave better resolution and a better sensitivity. A nitrogen–phosphorus detector for the sensitive GLC determination was also used [26] together with a packed column (column: OV-101 on Chromosorb W; isothermal). Clean-up was effected by partitioning into toluene. The data were similar to those obtained by a spectrophotometric procedure (Chinese standard method, including treatment with lead acetate). A similar spectrophotometric method was described by Chen [27].

In general, the non-chromatographic methods use hazardous chemicals to a greater extent (chloroform, lead salts and other heavy metals), and they are more tedious than, *e.g.*, HPLC methods. GLC methods can also be used, but an extraction is necessary. If only caffeine is to be determined an HPLC procedure after MgO treatment, filtration and dilution should be the method of choice. The pretreatment of the sample depends on what information is required. If one wants to determine the amounts of caffeine ingested by the consumer, a normal tea brew of unground material should be prepared, followed by a suitable clean-up prior to HPLC. The standard procedures normally determine the total extractable caffeine, so grinding and longer extraction times are included.

2.2. Volatile flavour compounds

As there are a large number of papers that deal with the composition of tea aroma and the analytical aspects, this section will only try to give some representative examples of the analytical concepts used. About 500 different aroma compounds have been identified in black tea [6,7,28,29]. According to Flament [29], there are 37 different hydrocarbons, 46 alcohols, 55 aldehydes, 57 ketones, 55 esters, 71 acids, 16 lactones, various furans, pyrroles and others. In recent years reviews on the composition and analysis of tea aroma have been compiled by, *e.g.*, Bokuchava and Skobeleva [30], Schreier [28] and Flament [29]; information on the biogenesis of the tea volatiles can be obtained from the same sources. Some aroma compounds in teas, such as alcohols and linalools, are biosynthesized directly by the tea plant (primary products); most of tea volatiles are formed during processing from precursors such as carotinoids, amino acids and lipids (secondary products) [6].

An aroma impact compound has not been identified as yet. Sanderson [31] stated in 1975 that, "The possibility that an as yet unidentified aroma constituent is the primary determinant of the character of black tea aroma is not entirely excluded".

Nowadays the method of choice is capillary GLC, as in analysis of flavours of other foods and beverages. Bokuchava and Skobeleva [30], who summarized the older attempts to determine tea aroma compounds, stated that there was no efficient method for tea aroma analysis before the introduction of GLC. The main effect on the results is due to the sample preparation used. A specific treatment according to volatility or the chemical nature of the compounds is necessary. This will be briefly outlined using some examples.

Heins *et al.* [32] employed a static headspace technique for identifying aroma components in dry tea leaves. They used GLC–MS, with the mass spectrometer disconnected during the injection of the (10-ml) vapour sample on to the capillary column (cooled by dry-ice). Using this method, 36 compounds from dry tea leaves were identified. Another headspace technique was published by Reymond *et al.* [33], who compared the fraction of highly volatile tea constituents. Wickremasinghe *et al.* [34] produced aroma concentrates of Ceylon black teas al-

ternatively by steam distillation and adsorption on charcoal of the headspace vapour over the tea brew and eluted with diethyl ether. The former method proved to be superior to the latter, which was partly due to artefact formation on the charcoal. Identification of the compounds was carried out by GLC and GLC–MS. As one result, variations in the aroma pattern of flavoury and non-flavoury tea were detected. A dynamic headspace technique combined with concentration of the volatiles was also used by Vitzthum and Werkhoff [35].

Solvent extraction procedures prior to GLC were used by other workers, *e.g.*, Yamanishi *et al.* [36]. They separated essential oils from black teas (isolated by solvent extraction using diethyl ether) by treatment with acidic and basic solutions, partitioned into a carbonyl-free and a carbonyl fraction and determined alcohols and carbonyls (such as benzaldehyde and phenylacetaldehyde).

Steam distillation was used, *e.g.*, by Yamanishi *et al.* [37]. They employed two traps for the steam volatiles (temperatures −15 and −78°C). The distillates were extracted with isopentane and diethyl ether and concentrated before analysis by GLC on packed columns. For the identification retention times, GLC–MS and IR were used; 57 compounds could be identified and their relative amounts were obtained. Similar procedures have been published (*e.g.*, 38–41]).

In most instances no real quantification of the steam volatiles was carried out. Relative amounts of the volatiles were obtained by calculating the ratio of peak areas and the internal standard area. Suitable internal standard compounds were, *e.g.*, ethyl caproate or ethyl decanoate.

Some workers (*e.g.*, refs. 42–44) use a so-called flavour index (FI). This is defined as the ratio of GLC peak areas (calculated *versus* an internal standard) of two groups of flavour compounds. The first group (VFC I) consists of the compounds with an undesirable flavour formed by twelve substances including hexanal, (*E,Z*)- and (*E,E*)-2,4-heptadienal,(*Z*)-3-hexenol,(*Z*)-3- and (*E*)-2-hexenal, *n*-pentanol and *n*-hexanol. The second group (VFC II), which imparts a sweet and flowery aroma to tea, includes twenty compounds such as linalool and its oxides, benzaldehyde, geraniol, α-terpineol, methyl salicylate, benzyl alcohol and β-ionone. Isolation is performed using SDE in a modified Likens–Nickerson apparatus. This concept was used to detect the influence of plucking on tea aroma [42], seasonal variations and the use of nitrogen fertilizers [43] or the effects of the altitude of the tea estate [44]. Similar approaches, such a terpene index, have also been made [45].

These analytical techniques have been applied in various studies on the influence of horticultural and technological variables such as plucking season and technique, use of fertilizers and withering on the formation of tea aroma. A Japanese group published a method for differentiating various tea clones by means of a terpene index [45].

Mick and co-workers [46–49] used successive extraction, vacuum distillation, liquid–liquid extraction with pentane and dichloromethane and column chromatography on silica gel to isolate four purified fractions of different polarity, which were analysed by capillary GLC on Carbowax or by GLC–MS. They also used GLC–MS to differentiate between two qualities of black tea (Darjeeling orange pekoe and Indian broken). A real quantification of some tea aroma components was performed.

A new aspect is due to the fact, that some volatile aroma constituents occur in form of enantiomers with different aroma values. Moreover, because of the different enantiomer patterns in natural and synthetically prepared aromas, adulteration can be detected. Werkhoff *et al.* [50] used enantioselective GLC on heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin in polysiloxane for the separation of *trans*-α-damascenone and *trans*-α-ionone enantiomers in tea and other foodstuffs.

According to Kinugasa and Takeo [51], who used GLC–MS (electron impact mode; column 50-m Carbowax 20M), the decomposition of unstable compounds (hexenal and esters) and the liberation of more volatile compounds (e.g., linalool and geraniol) from precursors lead to imbalances in aroma pattern and off-flavour.

Hence powerful methods for the analysis of tea volatiles are available. The problem is the assessment of the analytical data, because a generally accepted and convincing concept for the use of the data does not exist so far.

2.3. Polyphenols

2.3.1. Flavanols (catechins)

Polyphenols are the most abundant group of compounds in tea leaf. Among these, the flavanols (catechins) constitute the quantitatively major component, with up to 30% of the dry matter of fresh leaf. Fig. 1 gives the structures of the main flavanols of tea. Flavanols make an important contribution especially to the bitter and astringent taste of green tea. They are also of interest because of their physiological effects, in particular the capillary strengthening activity and antiartherosclerotic effect and the anticarcinogenic [52] and the bacteriostatic effects on several microorganisms. Acting as antioxidative agents or free radical scavengers, flavanols inhibit the Maillard reaction [53].

During the manufacture of black tea the flavanols are easily oxidized by polyphenol oxidase and further reactions lead to TFs and compounds of higher molecular mass. The amount and proportion of various flavanols, depending on the leaf age, are directly correlated with the quality of the final beverage. The finest teas are made from young tea shoots containing the highest flavanol levels.

Therefore, this group of tea constituents has been the subject of intensive studies for a long time. The first objective following the identification of the main flavanols of tea was to determine their distribution in different parts of the tea plant and their fate during the processing of black tea. For these investigations, *e.g.*, Roberts *et al.* [54] and Bhatia and Ullah [55] used 2-D PC after extracting the powdered green leaf with boiling methanol. After detecting the spots by their purple fluorescence under UV light in the presence of ammonia vapour, they were eluted with cold water and the absorbance of filtrates were measured at 275 nm.

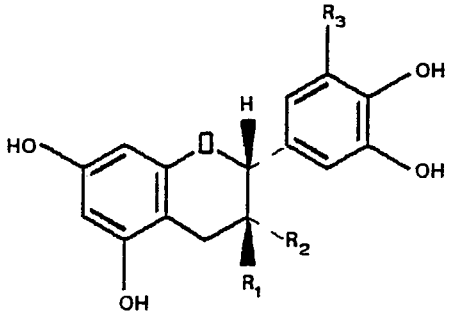

Fig. 1. Structures of flavanols in tea. R_1 = OH; R_2 = R_3 = H, (+)-catechin; R_1 = R_3 = H; R_2 = OH, (−)-epicatechin; R_1 = R_3 = H; R_2 = galloyl, (−)-epicatechin gallate; R_1 = H; R_2 = R_3 = OH, (−)-epigallocatechin; R_1 = H; R_2 = galloyl; R_3 = OH, (−)-epigallocatechin gallate.

2-D TLC was applied by Forrest and Bendall [56]. They used cellulose layers and developed in water or 5% aqueous methanol in the first direction and 1-butanol–acetic acid–water (4:1:5) in the second direction. Identification of polyphenols was carried out by means of co-chromatography, colour reactions and UV spectra. For quantification two different colour reactions were applied: (1) with benzidine–sodium nitrite–hydrochloric acid and (2) with vanillin–sulphuric acid, followed by measuring the absorbance at 450 and 500 nm, respectively. This method has been applied to flavanol analysis by several other groups [57–59]. TLC analysis on polyamide layers using different solvents and spray reagents for detection has also been described [60,61].

Because PC and TLC methods are time consuming and involve problems in quantification, GLC was introduced to overcome these disadvantages. In 1969 Pierce *et al.* [62] presented a method for the analysis of tea flavanols by GLC of their trimethylsilyl derivatives. Extraction of fresh or dried tea leaves with pyridine was followed by silylation with bistrimethylsilylacetamide (BSA). Two sets of isothermal conditions and a complex calibration procedure were necessary to separate and analyse the derivatives on a column packed with 3% OV-1 on 60–80-mesh Gas-Chrom Q. Collier and Mallows [63] improved the GLC method. They developed a temperature-programmed method for separation so that the complete analysis required only one run. Studies with different extraction solvents and conditions favoured ethyl acetate for selective and complete extraction of the flavanols from aqueous tea infusions. Solvent extraction is necessary to avoid interferences with other tea constituents. Similar GLC methods for the separation and determination of the flavanols of tea were applied by other workers [64–67].

Liquid chromatography was first employed for the preparative isolation of flavanols from green tea. Flavanols were extracted from tea leaves with boiling water and aqueous acetone and separated on Sephadex LH-20 columns [68–73]. Some appli-

cations of other stationary phases, *e.g.*, styrene–divinylbenzene copolymer or methacrylate esters [74], MCI gel CHP-20P or Bondapak C_{18} Porasil B [72] and high-porosity polystyrene gel Diaion HP20 [69], have also been described. The flavanol fractions were eluted with aqueous solutions of methanol, ethanol, acetone or their mixtures in varying compositions and monitored at 280 nm. This technique was also used for the isolation of minor flavanols in tea such as acetylated or 3-O-methyl flavanols [68,69,72].

Investigations on the effect of the extraction method on the determination of flavanols [75] showed that 90% aqueous methanol is the most efficient solvent compared with 90% ethanol, 90% acetone and water-saturated ethyl acetate. When comparing results from different methods, it is very important to consider the extraction technique.

Hoefler and Coggon [76] introduced HPLC with reversed phases into the analysis of flavanols in tea. For green tea the sample clean-up procedures could be reduced to a minimum. Even the direct injection of aqueous tea solutions after filtration through a membrane (cellulose, 0,45 μm) gave the desired separation. Moreover, the accurate and easy quantitative analysis inherent in GLC also applies in HPLC, which became the method of choice for the determination of non-volatile tea constituents. The authors used an RP-18 column (μBondapak C_{18}, 10 μm, 30 cm × 4 mm I.D.), flow-rate 2 ml/min and a UV detector with a 280-nm filter for separating the five flavanols EGCG, ECG, EGC, EC and catechin within 35 min. The mobile phase was acetic acid–methanol–DMF–water (1:2:40:157). This method has frequently been used for investigating green tea or monitoring the purity of preparatively isolated flavanols which were needed for model fermentation systems [77–80]. Several slightly modified HPLC methods have appeared [66,68,81–83]. Hirose and Tamada [81] analysed fresh green tea leaves using the described method with acetonitrile instead of methanol in the eluent. A Japanese patent [83] used acetone–THF–water (12:10:78). More differences could be found concerning the sample preparation, which should be mentioned in any case together with the chromatographic technique. Separation with gradient elution systems was carried out by Ma and Wang [84] and Liang *et al.* [85] with acetic acid–methanol–water (1:1:98) and acetic acid–methanol–DMF–water (1:1:50:48). However, the chromatograms showed no real improvement over isocratic elution.

A Japanese group developed another simple method for the determination of flavanols in infusions of green, oolong and even black tea by means of HPLC with gradient elution. Their solvents contained phosphoric acid, acetonitrile and DMF. A good separation within 35 min was achieved with an RP-18 column (Ultron-N C_{18}, 15 cm × 4.6 mm I.D.) held at 43°C [86]. As expected, the flavanol concentration in green teas was higher than in oolong and especially in black teas. Unfortunately, no HPLC separation of a black tea was published.

An RP-HPLC method for the determination of the main flavanols of tea together with caffeine, theobromine, gallic acid and theogallin has been reported recently [87]. For rapid and easy sample preparation an SPE technique with RP-18 cartridges was employed. The method is suitable for green, oolong and black tea and for tea extracts. A gradient method with eluents containing only 2% aqueous acetic acid and acetonitrile allowed a separation of the mentioned compounds in about 20 min. Fig. 2 shows a chromatogram of a green tea extract.

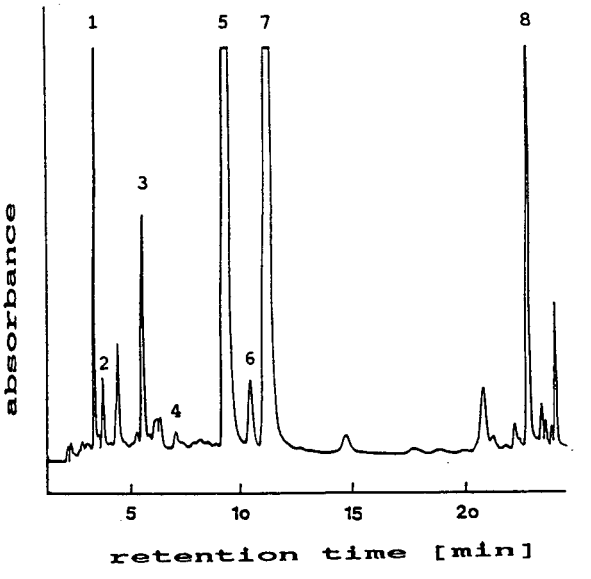

Fig. 2. HPLC profile of a green tea (measured by absorbance at 278 nm). 1 = Theogallin; 2 = gallic acid; 3 = EGC; 4 = catechin; 5 = caffeine; 6 = EC; 7 = EGCG; 8 = ECG. From ref. 87, with permission.

Comparison of different extraction methods, (1) a normal tea brew with boiling water, (2) a methanolic extract of ground tea and (3) the official German method for the determination of soluble solids (1 h boiling under reflux) [88], revealed a great influence on the flavanol yield. The official German method led to degradation of flavanols while methanolic extracts gave the highest yields. The use of DAD improved and simplified the confirmation of peak identity and purity [80,89], so that sample clean-up procedures became less important.

An interesting HPLC method using postcolumn derivatization with 4-dimethylaminocinnamaldehyde in the presence of concentrated sulphuric acid allowed the selective detection of flavanols [90]. A 200–40 000-fold sensitivity increase was observed for EC compared to other phenols. However, this sensitive method is time consuming and not necessary in analysis of the major flavanols of tea.

2.3.2. Flavonols and glycosides

The flavonols in tea are present as aglycones (traces) and to a much higher extent as their glycosides. In tea leaves all the flavonol glycosides derive from the aglycones myricetin, quercetin and kaempferol. Their structures are given in Fig. 3. The carbohydrate moieties, in most instances located at position 3 of the aglycone, consist of various combinations of glucose, galactose, rhamnose and, in a single instance, fructose. Mono-, di- and triglycosides have been observed. Flavonol glycosides are interesting compounds because of their positive physiological activities, especially their capillary

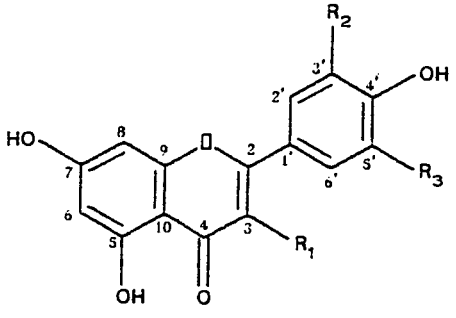

Fig. 3. Structures of flavonol and flavone aglycones in tea. R_1 = OH; R_2 = R_3 = H, kaempferol; R_1 = R_2 = OH; R_3 = H, quercetin; R_1 = R_2 = R_3 = OH, myricetin; R_1 = R_2 = R_3 = H, apigenin.

strengthening effect (so called vitamin P activity). Owing to their yellow colour, the flavonol glycosides contribute to the colour of the tea brew in addition to TRs and TFs. Moreover, the colour of the green tea brew is caused almost exclusively by flavonol glycosides. For this reason, most of the investigations on flavonol glycosides deal with green tea or fresh tea shoots.

The first investigations on flavonols and their glycosides in tea were carried out by the groups of Roberts and Oshima and Nakabayashi. The Japanese workers [91] separated 23 flavonols, flavones and their glycosides by means of 2-D PC from extracts of tea leaves (var. *assamica*). Nine of them could be identified as derivatives of kaempferol and some others as glycosides of quercetin, including rutin. For the latter they published a spectrophotometric method for its semiquantitative determination [92]. They also used column chromatography on magnesium or zinc silicate for isolation. Roberts *et al.* [93] used 2-D PC to separate the flavonol glycosides and aglycones from Indian fresh tea leaves and black tea. Dried tea was ground and three volumes of water were added. The extract was centrifuged and applied to the paper. Chromatograms were run with 1-butanol–acetic acid–water (4:1:2.2) in the first direction followed by acetic acid (2%). This chromatographic system became a standard system for the separation of flavonoids from tea and was used frequently during the next few decades. Roberts *et al.* [94] also applied this system to taxonomic investigations on different camellia species. Spot detection was carried out either by fluorescence in UV light or for sufficiently high concentrations, by spraying with iron(III) chloride–potassium hexacyanoferrate(III) reagent, which gave blue reaction products with quercetin and myricetin derivatives. Acetic acid (2%) as the second solvent could be replaced with some advantage by 2% aqueous boric acid. This did not lead to any changes in R_F values but, owing to the formation of borate complexes, most of the flavonols showed up as bright yellow spots intensifying under UV light without any spray reagent being necessary. Some flavonol glycosides that remained unresolved under these conditions could be separated using 80% aqueous phenol followed by 1-butanol–acetic acid–water as solvent. Imperato [95] isolated three flavonol glycosides from a tea brew using preparative PC employing

mixtures of 1-butanol–acetic acid–water (4:1:5, upper phase), 5% acetic acid and 1-butanol–ethanol–water (4:1:2.2) as solvents.

The Georgian team of Chkhikvishvili and co-workers [96,97] used column chromatography and TLC for the isolation and separation of various flavonoids from tea. Generally, a clean-up of the extract prior to analysis, *e.g.*, by PC, TLC, UV, ^{1}H NMR or MS, is essential. Suitable clean-up techniques are column chromatography on silica gel [96,98], polyamide [96,99] or cellulose [100] and gel chromatography on Sephadex LH-20 [96,100,101]. A procedure for the separation of flavonoids from tea was described [98]: tea shoots were extracted with chloroform–benzene (1:1) to remove lipophilic substances and caffeine. Flavonoids and catechins were extracted with ethyl acetate. Catechins were removed from this extract by column chromatography on silica gel (washing with diethyl ether) and the residual flavonoids were desorbed with methanol. The eluent was analysed by TLC on polyamide and silica gel layers. Ten individual compounds (flavonol glycosides, flavones, flavanones and dihydroflavonols) were identified.

None of these methods allowed the determination of flavonols or flavonol glycosides. The first intensive quantitative study on flavonol glycosides from tea was performed by Bokuchava and Ulyanova [102]. Flavonol glycosides were extracted with methanol from unprocessed tea leaves, which had been previously treated with chloroform to remove caffeine. Ammonia solution was added to the methanolic extract (pH 10) to force the destruction of the bulk of catechins and tannins by oxidation. Oxidation products were separated from the solution by filtration and the filtrate was analysed by 2-D PC using 1-butanol–acetic acid–water (40:12:29) and 2% aqueous acetic acid as solvents. Slices of the chromatogram were sprayed with $AlCl_3$ (1% ethanolic) to detect flavonol glycosides. The compounds of interest were eluted from the paper using ethanol (50%, aqueous) and determined spectrophotometrically. Owing to the alkaline treatment, myricetin glycosides, which are very sensitive to oxidation, could not be determined by this method.

In the last few years, HPLC has been introduced as the method of choice for flavonol analysis of tea. Detection was carried out using a UV detector or, with some advantage, by DAD. Tsushida *et al.* [103] obtained flavonol aglycones by hydrolysing a tea extract with 1.2 *M* HCl for 30 min under reflux, followed by ethyl acetate extraction. They separated the three aglycones myricetin, quercetin and kaempferol by HPLC on RP-18 material using a phosphate buffer as eluent. Absorbance was monitored at 325 nm, which is not in accordance with the UV maxima of flavonol aglycones at *ca.* 270 and 370 nm. HPLC analysis of flavonol glycosides from tea was introduced by Biedrich *et al.* [104]. Flavonol glycosides were isolated from black tea by column chromatography on polyamide. An RP-HPLC method with gradient elution [solvents: (A) 2% aqueous acetic acid, (B) acetonitrile] was developed for separation. By means of the UV spectra obtained from DAD, several peaks could be identified as flavonol glycosides. The authors concentrated on the determination of rutin (quercetin 3-O-rhamnoglucoside), which could be identified by co-chromatography. McDowell *et al.* [105] used the RP-HPLC system and method of Bailey *et al.* [89] to study the contribution of flavonol glycosides to the colour of black tea liquors. From chromatograms of the entire tea brew they estimated that 48% of the absorption at 380 nm was caused by flavonol glycosides, 25% by TFs and the remainder by the so-called TRs (*cf.*, section 2.3.6). Liang *et al.* [85] developed an HPLC method to separate and determine several groups of tea constituents, including flavonols. The analytes were extracted from tea with ethanol (non-phenolic pigments had been removed previously with light petroleum). The extract was cleaned up using a Sep-Pak column and analysed by HPLC on a μBondapak fatty acid column using 55% aqueous methanol adjusted to pH 3.0 with phosphoric acid. The detection wavelength was 254 nm. Seven peaks appeared during the first 5 min, three of which were identified by co-chromatography (quercetin, myricetin and rutin). Although the separation was very poor, quantification was carried out. The amounts of, *e.g.*, myricetin (up to 1.67 mg/g) in Chinese green teas were much higher than reported by other workers. As no further confirmation of peak identity and purity were carried out, the results obtained by this method seem to be unreliable. Recently, Engelhardt and co-workers [106–108] published a method for the HPLC determination of flavonol glycosides. Analytes were extracted with methanol and aqueous methanol and cleaned up by column

chromatography on polyamide (washing with water, elution with methanol). HPLC separation of the eluate was done by two isocratic systems: 2% aqueous acetic acid–acetonitrile (85:15) and 2% aqueous acetic acid–1,4-dioxane–methanol (77:13:10) on an RP column (ODS-Hypersil). Chromatograms were monitored by DAD at 354 nm (Fig. 4). Fifteen flavonol glycosides were isolated by means of preparative HPLC and identified by various methods such as UV, ^{1}H and ^{13}C NMR, MS, hydrolysis and analytical HPLC and GLC. The method was applied to the determination of flavonol glycosides in 30 samples of black, green, oolong and instant teas [106].

2.3.3. Flavones and glycosides

In addition to flavonol glycosides, tea contains several flavone glycosides in lower amounts. The structure of the most important aglycone in tea, apigenin, is shown in Fig. 3. In contrast to flavonol glycosides, which have O-linked carbohydrate moieties, flavone glycosides in tea appear nearly exclusively as C-glycosides. Isolation and identification techniques are similar to those applied to flavonol glycosides. Moreover, C-glycosides cannot be hydrolysed under normal conditions. This causes some problems in the identification of the sugar moieties of tea flavone glycosides. However, it facilitates the separation of flavone glycosides from the bulk of flavonol glycosides.

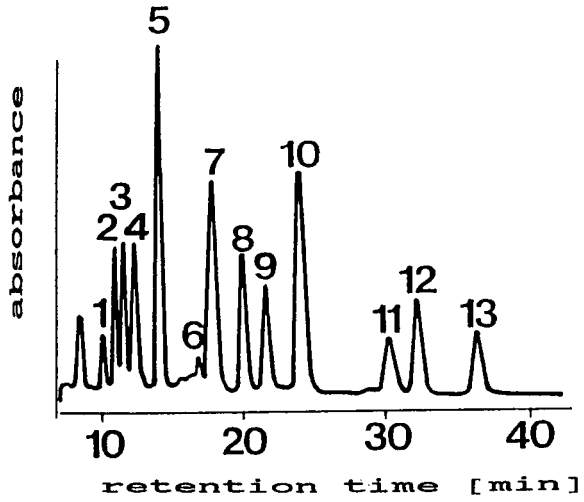

Fig. 4. HPLC separation of black tea flavonol glycosides (Sikkim Temi first flush). Chromatogram at 354 nm. 1 = Myricetin-3-O-rhamnoglucoside; 2 = myricetin-3-O-galactoside; 3 = myricetin-3-O-glucoside; 4 = quercetin-3-O-glucorhamnogalactoside; 5 = quercetin-3-O-rhamnodiglucoside; 6 = quercetin glycoside; 7 = quercetin-3-O-rhamnoglucoside and kaempferol-3-O-glucorhamnogalactoside; 8 = quercetin-3-O-galactoside; 9 = quercetin-3-O-glucoside; 10 = kaempferol-3-O-rhamnodiglucoside; 11 = kaempferol galactoside; 12 = kaempferol-3-O-rhamnoglucoside; 13 = kaempferol-3-O-glucoside. From ref. 108 with permission. Identification of peaks 4 and 7 according to ref. 106.

There are only a few papers on flavone glycoside analysis in tea. Some of them have been mentioned above (see Section 2.3.2). The first report on flavones in tea was published by Sakamoto [109]. He observed nineteen flavones in a green tea infusion. Separation was carried out by 2-D PC [solvents: (1) 1-butanol–acetic acid–water (4:1:2); (2) 2% aqueous acetic acid]. IR and UV spectra were recorded after elution of the spots. The identification of some of the compounds was described in a later paper [110]. Chauboud *et al.* [111] used a similar solvent system for the isolation of another flavone glycoside from tea. Apigenin di-C-glycosides obtained from tea by the method of Sakamoto [109] were separated using HPLC by Tsushida *et al.* [103]. The detection wavelength was set at 325 nm. Recently, Engelhardt *et al.* [112] worked on flavone glycosides from black tea. Flavone glycosides were extracted together with flavonol glycosides (see section 2.3.2) by methanol. The eluate obtained from column chromatography on polyamide was refluxed with HCl to hydrolyse the flavonol O-glycosides. The hydrolysate was cleaned up again using a polyamide SPE cartridge and analysed by HPLC (ODS-Hypersil, gradient elution with 2% acetic acid and acetonitrile, DAD). Fig. 5 shows the HPLC trace of the

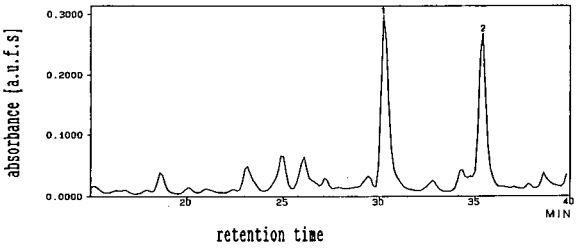

Fig. 5. HPLC trace of a hydrolysed tea brew (Sikkim Temi). Column, Hypersil-ODS (5-μm); solvents (A) 2% aqueous acetic acid, (B) acetonitrile, gradient 90% A, in 15 min to 85% A, 5 min isocratic, in 10 min to 70% A, 10 min isocratic; flow-rate, 1 ml/min; detection at 340 nm. 1 = Vitexin; 2 = isovitexin. From ref. 112, with permission.

flavone glycosides from a black tea sample monitored at 340 nm. The major drawback of acid hydrolysis is due to a Wessely–Moser rearrangement leading to isomerization of unsymmetrically substituted flavones (*e.g.*, vitexin and isovitexin). For that reason, quantitative data could only be presented for the sum of the amounts of those isomers.

2.3.4. Phenolic acids and esters

The major representatives of this group of tea constituents are gallic acid and its tea-specific ester with quinic acid (theogallin) and hydroxycinnamoyl acid/quinic acid esters (depsides).

2.3.4.1. Free gallic acid. Gallic acid (Fig. 6) is the most important phenolic acid in tea. The amount of gallic acid increases during the fermentation owing to its liberation from catechin gallates. Oshima and Nakabayashi [113] described a spectrophotometric method for its determination in tea using HCl–formaldehyde and alkaline phosphomolybdic acid.

Determination by means of HPLC is more specific than spectrophotometry. Sample clean-up does not seem to be essential, as most workers employed direct injection of a tea brew after filtration through a membrane filter. In general the separation is carried out on RP-18 columns. One of the first HPLC applications to tea analysis was that of Hoefler and Coggon [76], who separated a number of tea components including gallic acid using a μBondapak C_{18} column (10 μm) and a fixed-wavelength UV detector (254 nm). The mobile phase was 0.02 *M* citrate–phosphate buffer (pH 4.5).

More convenient is the use of non-salt-containing mobile phases as done by Ma and Wang [84]. The gradient elution solvents contained water–acetic acid–methanol (98:1:1) and DMF–water–acetic acid–methanol (50:48:1:1). They used a Micropak SP-C_{18} column (5 μm) and a UV detector set at 280 nm, which led to an increase of sensitivity. Gallic acid contents in brews obtained from Chinese tea samples ranged from 0.4 to 1.6 g/kg dry mass. These results correspond fairly well with Engelhardt's [114] results (0.9–5.5 g/kg dry mass). He also used direct injection of a tea brew on a Nucleosil RP-18 column with an isocratic system [2% aqueous acetic acid–acetonitrile (90:10)]. Recently, Bailey *et al.* [89] published investigations on non-volatile tea constituents. They detected several compounds in a black tea liquor, including gallic acid, using DAD. Separation was carried out on a 5-μm Hypersil-ODS column by a linear gradient of 2% aqueous acetic acid and acetonitrile. Only a single sample was analysed and no quantification was done.

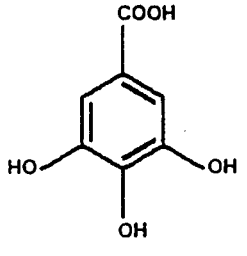

Fig. 6. Structure of gallic acid.

Kuhr and Engelhardt [87] developed an RP-HPLC method for the determination of gallic acid together with flavanols, theogallin and caffeine in tea employing solid-phase extraction on RP-18 cartridges for the clean-up procedure, as discussed in Section 2.3.1. Fifteen black tea and two each of green, oolong and instant tea samples were analysed. Gallic acid contents ranged between 0.6 and 6.2 g/kg dry mass in tea samples. They also studied different techniques for gallic acid extraction. The yield of a methanolic extraction was higher than that of a normal tea brew. They noticed a considerable increase in gallic acid during extraction when using the official German method (refluxing with water for 1 h), probably caused by depolymerization/decomposition of, *e.g.*, catechin gallates or TRs.

2.3.4.2. Theogallin. Although theogallin is a particularly interesting compound, as it specifically appears in tea, only few investigations have been published on this subject. Theogallin was isolated and characterized as galloylquinic acid by Roberts and Myers [115]. They used a Craig distribution system, cellulose acetate column chromatography and preparative PC for isolation. The purity of the isolate was monitored by 2-D PC [eluents: (1) butanol–aqueous acetic acid; (2) aqueous acetic acid]. The structure of theogallin (Fig. 7) was finally assigned to be 5-O-galloylquinic acid [116] by NMR analysis.

Bhatia and Ullah [117] observed a decrease in theogallin content during tea fermentation. They also used 2-D PC followed by photometric measure-

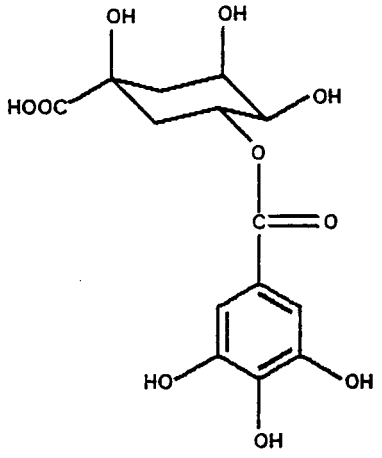

Fig. 7. Structure of theogallin.

ment of the spots at 275 nm. Unfortunately, the solvent composition used was not given. An HPLC method for determination of theogallin has been mentioned and described above [87] (see Section 2.3.1).

2.3.4.3. Chlorogenic acids and p-coumaroylquinic acids. Derivatives of hydroxycinnamic acids are widely distributed in the plant kingdom. Caffeoyl- and *p*-coumarylquinic acids (CQAs and CouQAs, respectively, see Fig. 8) have been described in tea. It should be noted that a different (non-IUPAC) nomenclature was used in the earlier literature.

Early investigations on hydroxycinnamic acid esters in tea were carried out by Roberts [118]. A black tea infusion was prepared and extracted with ethyl acetate. Analysis of this extract by means of 2-D PC led to the detection of three isomers each of

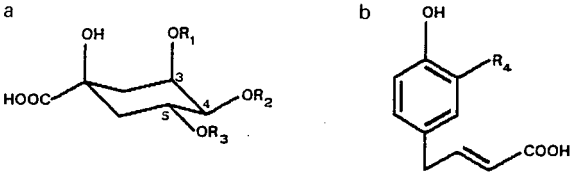

Fig. 8. Structures of *p*-coumaroyl- and caffeoylquinic acids. Numbering according to IUPAC rules. (a) R_1 = X (see b); R_2 = R_3 = H, 3-CouQA; R_1 = R_3 = H; R_2 = X, 4-CouQA; R_1 = R_2 = H; R_3 = X, 5-CouQA; R_1 = Y; R_2 = R_3 = H, 3-CQA (neo-chlorogenic acid); R_1 = R_3 = H; R_2 = Y (see b), 4-CQA (crypto-chlorogenic acid); R_1 = R_2 = H; R_3 = Y, 5-CQA (*n*-chlorogenic acid). X = *p*-coumaric acid (R_4 = H); Y = caffeic acid (R_4 = OH).

CQA and *p*-CouQA among several other phenolic compounds. In a later paper, Roberts and Williams [119] reported the UV spectra of 5- and 3-CQA measured after elution from the paper and that of CouQAs which were obtained by direct measurement on the paper.

Other techniques used for hydroxycinnamic acid isolation were preparative PC, TLC and column chromatography on cellulose, silica or polyamide phases. A method for the separation and determination of isomers of CQAs in tea was developed by a Chinese group [120]. The analytes were extracted by 20% aqueous acetic acid for 2 h, followed by membrane filtration. For separation they used a μBondapak C_{18} column, 20% aqueous acetic acid as mobile phase and a UV detector set at 324 nm. Peak identification of neo-, crypto-, *n*- and isochlorogenic acid was done by co-chromatography. Isochlorogenic acid means all possible isomers of dicaffeoylquinic acid. Unfortunately, peak identification was not confirmed by, *e.g.*, co-chromatography using an additional HPLC system. All four compounds mentioned above appeared in the first 3 min of the run. Contents in black tea were in the ranges 134–156 mg per 100 g (neo-CQA), 268–281 mg per 100 g (crypto-CQA), 67–99 mg per 100 g (*n*-CQA) and 99–109 mg per 100 g (iso-CQA).

Engelhardt *et al.* [121] obtained CQAs and CouQAs from a tea brew. Different clean-up procedures prior to HPLC analysis, such as ultrafiltration, polyamide column chromatography and SPE with RP-18 cartridges, were compared with direct injection of the tea brew. The SPE clean-up gave the best results. Owing to the high polarity of the CQAs and CouQAs, they can be eluted even with water, which makes this clean-up very simple. Fig. 9 shows an HPLC trace of a black tea brew treated in this way. Peak identity and purity were confirmed by UV spectra obtained with DAD. The appearance of mono-CQA and CouQAs in tea could be confirmed. However, this technique could not verify the identification of dicaffeoylquinic acids. Comparison of the results obtained from mono-CQA determinations in black tea with the results in ref. 120 shows a discrepancy which is higher than the natural range of contents that could be expected for different samples and the different extraction modes. In the Chinese work [120], the determination may have included some co-eluting compounds.

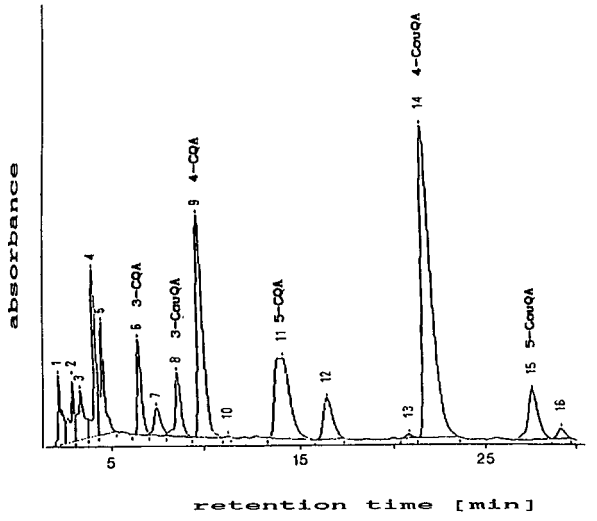

Fig. 9. HPLC separation of CQA and CouQA from a black tea brew, cleaned up by SPE. From ref. 121, with permission.

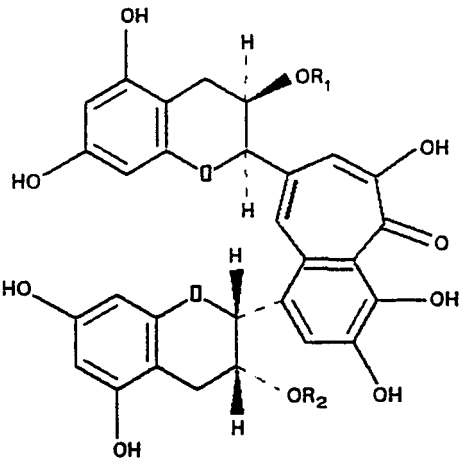

Fig. 10. Structures of the major theaflavins in tea. $R_1 = R_2 = H$, theaflavin; $R_1 = H$; R_2 = galloyl, theaflavin-3-gallate; R_1 = galloyl; $R_2 = H$, theaflavin-3′-gallate; $R_1 = R_2$ = galloyl, theaflavin-3,3′-digallate.

2.3.5. Theaflavins

During the fermentation stage of black tea processing, catechin or EC and their gallates are involved in an enzyme-catalysed oxidative reaction to form the so-called TFs [122]. These compounds, containing a benzotropolone group, are orange-red, constitute about 0.3–1.8% of black tea (dry mass base) and contribute significantly to the bright colour and brisk taste of tea brews [123]. Pure TFs in aqueous solution are normally very astringent but in tea the astringency is reduced owing to an interaction with bitter caffeine. Fig. 10 shows the formulae of the major TFs. Theaflavic acids are the oxidation products of an analogous reaction with the quinones of flavanols and gallic acid. Several attempts have been made to find correlations between TF contents, results of tea tasters and the quality of tea [124,125], but this is still controversial. After isolation by means of 2-D PC and gel chromatography the structures of the various TFs could be elucidated by MS, NMR and IR techniques [54,126–128].

The commonly used spectrophotometric method for the determination of total TFs in tea infusions was developed by Roberts and Smith [129,130] and modified by Ullah [131] for climatic conditions in subtropical countries. After extracting the TF from hot aqueous tea brews with ethyl acetate or IBMK and measuring the absorbance at 380 and 460 nm, respectively, the total TF amount was calculated based on mean molar absorptivities. However, later investigations showed that these values depend on the composition of the TF fraction. This easy and rapid method was mostly employed for investigating the influence of different processing parameters, *e.g.*, time and temperature of fermentation [117]. Hilton and Ellis [124] introduced another spectrophotometric method involving flavognost reagent (2-aminoethyl diphenylborate in ethanol), which forms a green complex with the *cis*-1,2-dihydroxybenzene ring associated with the TFs. In general, this rapid method gives reproducible results if the conditions of several critical stages [132–135] such as particle size of ground material, temperature of water used and infusion time are well defined and under control. The method has also often been applied to studies on the effects of different parameters on processing of tea and the resulting tea quality [125,136] and to investigations of the kinetics of tea infusion [134,135,137–139].

Other non-chromatographic methods for TF analysis were described by Fernando and Plambeck [140], who used voltammetric methods, and Hall *et al.* [141], who applied NIR spectrometry. However, these methods did not become common because the required analytical equipment is often not available. The limitation of all of these non-chromatographic methods arises from their inability to sep-

arate the mixture of TFs into its individual components, so that only values for the total amounts of TFs can be obtained.

The purpose of Collier and Mallows' work [142] was to develop a more specific and reproducible method. They reported a method for separating and determining the TF by temperature-programmed GLC of their trimethylsilyl ethers. Fractions suitable for analysis were prepared from whole tea solubles by extraction with ethyl acetate and chromatography on Sephadex LH-20. TF could be separated from the monogallates and the digallate in 25 min on columns packed with 3% OV-1 on Chromosorb W, but the two isomeric monogallates were not resolved. The results obtained by this GLC method were lower than those given by Roberts' spectrophotometric method. The authors explained this result by incomplete extraction of TRs from solutions in ethyl acetate in Roberts' method. Another possible reason will be discussed later.

By means of column chromatography on Sephadex LH-20 with gradient elution of aqueous acetone from 40 to 80%, Takeo [143] was able to separate TF, TF monogallates (the two isomers were not resolved) and TF digallate. The contents of three TFs were determined spectrophotometrically from the molar absorptivity of authentic TF at 460 nm.

An HPLC separation of TFs on RP material was published by Hoefler and Coggon [76] and led to remarkable improvements. The separation was performed on a μBondapak C_{18} (10-μm) column. The mobile phase was acetic acid–acetone–water and UV detection (365-nm filter) was used. With a slightly modified version of this method (380-nm detection wavelength, different eluent proportions) the changes undergone by tea polyphenols during storage of tea brews were investigated [144]. Robertson [78,79] and Robertson and Bendall [145] used a modification of Hoefler and Coggon's method for the HPLC analysis of TFs formed from flavanols in a model fermentation system under various conditions. Bajaj *et al.* [146] analysed the behaviour of TFs themselves in a model oxidation system with a similar method.

Wellum and Kirby [147] systematically examined the factors that could be important in TF analysis using HPLC: sample preparation, column temperature, the use of a ternary solvent system and solvent gradient programming. Finally, they prepared the sample by means of solid-phase extraction using Sep-Pak C_{18} cartridges with TF recoveries of 98–100%. Separation on Partisil ODS columns (5 or 10 μm) was performed at 80°C applying a gradient elution system with acetone–water–methanol. Nevertheless, the two isomeric monogallates remained unresolved. Later, Anan *et al.* [148] succeeded in separating them. They also used an RP column [ODS-120A, 10 μm (Toyo Soda), 250 × 4.6 mm I.D.], elevated temperature (57°C) and detection at 375 nm, but a mobile phase containing phosphoric acid in addition to water and acetone. Sample preparation consisted only of extracting black tea with 40% aqueous acetone for 30 min, adding water, centrifuging and filtering the supernatant. The recovery of TFs was between 91.1 and 96%.

An isocratic RP-HPLC method and the flavognost method were compared in determining the TFs [149]. The HPLC method is more time consuming owing to the need for preseparation by gel chromatography, but is able to separate the four TFs (see Fig. 11) and determine their relative amounts. In general, the results obtained by the flavognost method were considerably higher than the HPLC results.

The use of DAD in the HPLC analysis of black tea [89] revealed the presence of interfering substances (flavonol glycosides) in fractions used to determine TFs and TRs in the spectrophotometric analysis developed by Roberts and Smith [129]. The contribution of flavonol glycosides to the absor-

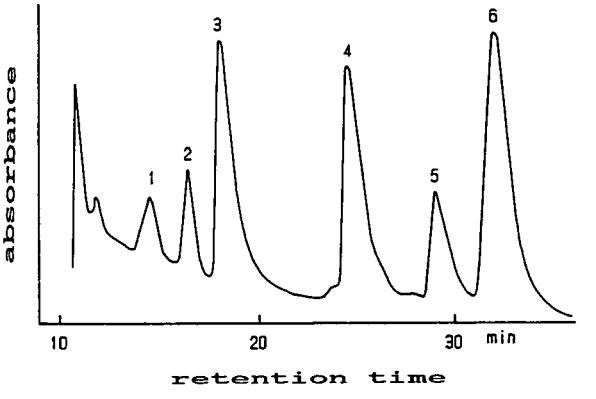

Fig. 11. HPLC separation (375 nm) of theaflavins in black tea. 1 = Isotheaflavin (tentative); 2 = epitheaflavic acid; 3 = TF; 4 = TF-3′-gallate; 5 = TF-3-gallate; 6 = TF-3,3′-digallate. From ref. 149, with permission.

bance at 380 nm is about 33%. Therefore, Roberts and Smith's method always led to an overestimation of TFs [105]. A recently published method took this fact into account. The authors used C_{18} sorbent cartridges and stepwise elution of TRs and TFs with acidified methanol for sample preparation and measured the absorbance of different fractions at one or more of the wavelength settings 380, 440 or 460 nm [150]. A comparison with HPLC, NIR and spectrophotometric methods using flavognost and aluminium chloride reagents and Roberts and Smith's method was also described. Nevertheless, in the analysis of TFs the peak identity should be verified with photodiode-array detection as described in recent publications [149,151] to avoid misleading results. Recently a comparative study of RP-HPLC of black tea phenolic pigments resulted in improved chromatograms concerning resolution and peak shape using a Hypersil ODS column (5 μm) with a citrate buffer [152]. The chelating agent citric acid reduced secondary retention through removal or masking of surface metals. Eight TFs and three theaflavic acids could be separated for the first time in one chromatogram monitored at 460 nm.

2.3.6. Thearubigins

Thearubigins (TRs) is the name originally assigned to a heterogeneous group of orange-brown, weakly acidic pigments formed by enzymic oxidative transformation of flavanols during the manufacture of black tea [122]. TRs have been claimed to be the most abundant polyphenolic fraction of black tea contributing significantly to taste, depth of colour and body of a tea brew and therefore influencing the quality. Roberts *et al.* [54] first attempted to separate this complex mixture of substances by means of 2-D PC and by liquid–liquid partitioning. They classified the TRs into three fractions: the SI-TRs extractable into ethyl acetate, the SII-TRs remaining in the aqueous phase and the SIa-TRs also in the aqueous phase but being more soluble in diethyl ether. Using successive extraction of tea liquor with ethyl acetate and *n*-butanol followed by fractional precipitation, Brown *et al.* [153] isolated five TR fractions, each being degradable into anthocyanidins, gallic acid or flavanols under different conditions of hydrolysis. For that reason TRs were assigned to be polymeric proanthocyanidins containing flavonoid residues. Cattell and Nursten [101,154] examined the relatively low-molecular-mass fraction SI soluble in ethyl acetate by means of gel chromatography on Sephadex LH-20 and 2-D PC. They obtained three subfractions, each having an M_r of about 1500. After degradation only small amounts of anthocyanidins were obtained and it was concluded that these TR fractions are pentameric flavanols with hydrolysable and non-hydrolysable links and benzotropolone units.

Several attempts have been made to separate the TRs by means of gel chromatography often in combination with 2-D PC [143,155–159]. However, many of these so-called "separations of TRs" were in fact separations of low-molecular-mass compounds accompanying the TRs, especially when whole tea brews were analysed. Many of these misleading results were obtained because of adsorption effects which disturbed the SEC. In other instances only poor separations could be achieved when analysing single TR fractions. Most promising was the application of the porous polymer packing material Toyopearl [157] for gel filtration, which showed a much higher resolving power than the frequently used Sephadex LH-20. However, chromatography of TRs is difficult, because they consist of compounds with a wide molecular mass range (700–40 000) [160] and strongly adsorb on active surfaces. Another method for isolating TR fractions was carried out by Wedzicha and Lo [161] using a multilayer coil planet centrifuge for counter-current chromatography. They obtained pure SII and a mixture of SI and SIa TR in a relatively short time (1–2 h).

The most frequently used method for the determination of TRs is that of Roberts and Smith [129] already mentioned in Section 2.3.5, measuring the absorbance of distinct solutions at 380 nm [42,44,117,130,159,162–168] or a modified version [124,131,143,169,170]. McDowell *et al.* [105] checked this method with HPLC and revealed that TRs contribute only about 23% to the absorption of the sample solution at 380 nm, whereas flavonol glycosides account for 72% and TFs for 5%. It is obvious that quantitative measurement based on absorbance at particular wavelengths can only result in approximate values for such an inhomogeneous group of compounds, especially when these substances could not be completely separated from others. A recently developed spectrophotometric

method measured absorbance at 380, 440 and 460 nm after sample preparation using C_{18} cartridges and stepwise elution with acidified methanol [150]. The selectivity of this method for separating TR and TFs was assessed by analysing the different fractions by means of HPLC with DAD.

A different way to elucidate the TR formation and structure is the method of Robertson [78,79] and Robertson and Bendall [145] using a model fermentation system. The products formed during *in vitro* enzymatic oxidation of flavanols under controlled conditions were analysed by HPLC. They used an RP-C_{18} column (Hypersil ODS, 5 μm) (20 cm × 0.4 cm I.D.) and a mobile phase consisting of 29% aqueous acetone containing 1% acetic acid. In the HPLC elution profile monitored at 375 nm they could identify the TFs among separated peaks and a number of compounds also appeared in green tea extract. The remainder was summarized under the name TRs. With a similar HPLC method Roberts *et al.* [144] carried out a study of changes undergone by TRs and TFs during storage of tea brews. From the HPLC profiles the increase in the TR fraction was determined.

To overcome the problems with the high affinity of TRs for stationary phases such as RP-C_{18} material, Wedzicha and Donovan [171] checked the potential applicability of normal-phase HPLC of derivatized SI-TR. After extraction from black tea infusions and inspection to be free from low-molecular-mass impurities, SI-TRs were converted into acetyl and methyl derivatives. The best separation of the more stable methylated SI-TRs by HPLC was obtained using a silica phase (Partisil 10) (25 cm × 0.46 cm I.D.) with a chloroform–methanol gradient, but no further identification was done.

The combination of HPLC with DAD provided additionally spectral information on each resolved component. Based on the method reported by Robertson and Bendall [145], Opie and co-workers [80,172] used the model fermentation system together with HPLC and DAD on 3-μm RP-18 particle packings to improve separation. They also replaced acetone with acetonitrile, which is advantegeous in obtaining spectra in the UV region. More than 40 pigments were separated from untreated black tea liquors and four of them were identified as TFs (see Fig. 12). Most of the other peaks showed absorption maxima between 390 and 410 nm, *i.e.*, in the yellow-pale orange region. Changes in TR profile during fermentation or substances produced by *in vitro* oxidation of a standard catechin mixture could be observed and compared by means of this method.

A comparative study of different types of RP materials (Hypersil-ODS, Hypersil octyl wide pore, Hamilton PRP-1) for the analysis of black tea pigments has recently been published [152]. The best resolution was obtained on a Hypersil-ODS column with a citrate buffer acting as a chelating agent for removing or masking surface metals. The pigments were classified by their chromatographic behaviour into three groups: pigments which ran close to the void volume of the HPLC columns because of size

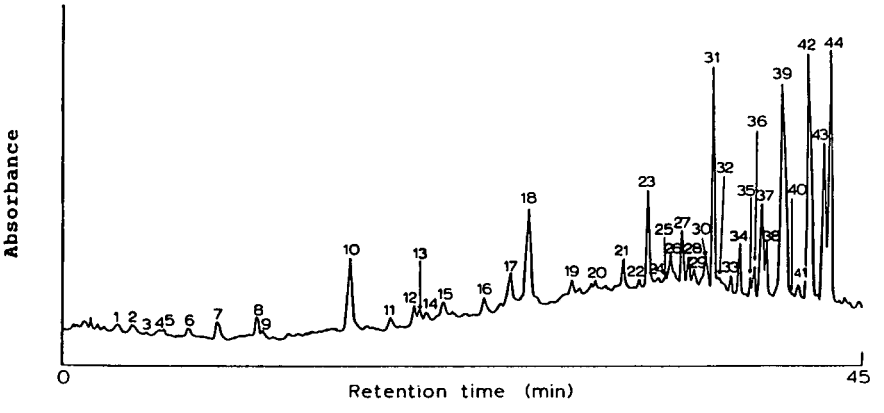

Fig. 12. HPLC analysis of a whole black tea liquor (450 nm). From ref. 80, with permission.

exclusion, resolved pigments and unresolved pigments. The resolved pigments were further classified by DAD UV–VIS spectra into TFs, TF acids and resolved TRs. The unresolved components, which were eluted as a broad, convex band on the wide-pore column and the Hamilton column (styrene–divinylbenzene copolymer), showed absorption acrosss the entire spectral region, indicating that they were brown pigments, possibly polymeric TRs.

It should be stressed that only chromatographic methods can improve our knowledge of TRs. Classical methods overestimate TR contents and so data have to be revised.

2.4. Miscellaneous

2.4.1. Non-phenolic pigments

There are two major groups of non-phenolic pigments which contribute to the colour of tea leaves, chlorophylls and its degradation products (pheophytins, pheophorbides) and carotenoids. Most of the papers on non-phenolic pigments deal with both groups. Therefore, we shall discuss them together in this section. Until recently, only PC and TLC had been applied to non-phenolic pigments of tea. Subsequently HPLC has also been introduced into this field.

Vachnadze *et al.* [60,173] used TLC for the separation of chlorophyll from a crude extract of tea catechins (solvent: ethanol). This extract was monitored for chlorophyll by means of TLC on a silica gel layer which had been impregnated with formamide. Light petroleum–formic acid (10:1) was employed as a solvent. Five chlorophyll spots were observed under UV radiation. A method for the determination of chlorophylls and accompanying pigments such as carotenoids from tea by means of TLC–spectrophotometry was established by Dzhindzholiya and Revishvili [174]. Tea was macerated with acetone, $CaCO_3$ and Na_2SO_4. The mixture was filtered and separated by TLC on a Silufol layer with light petroleum–ethanol (16:1) as the mobile phase. Determination by means of spectrophotometry was carried out after elution of the carotene, chlorophyll and carotenoide spots with light petroleum, diethyl ether and ethanol, respectively. Dev Choudhury and Bajaj [175] reported a solvent system for the separation of chlorophylls, their derivatives (pheophorbides and pheophytins) and carotenoids in tea. A Chinese researcher [61] developed a rapid method for the determination of chlorophylls and carotenoids by 2-D TLC. He employed acetone as a solvent extraction. The compounds were separated on polyamide using ethanol–water (1:1.1) in the first and toluene–ethanol–water (2:6.8:1) in the second direction. Detection was done by spectrofluorimetry. In addition to several catechins, chlorophyll *a* and *b*, xanthophyll and β-carotene were detected and identified. Komiya *et al.* [176] described a number of methods to determine tea constituents, including chlorophyll, by means of spectrophotometry. The chlorophyll concentration was very low in all samples.

TLC analysis of unsaponifiable matter from various seed oils including tea seed was performed by Nasirullah and Kapur [177]. The $CHCl_3$ solution of the hydrolysed oil was separated with benzene–ethanol (94:6). β-Carotene and several other compounds were identified. A rapid method for the extraction and TLC separation of tea leaf carotenoids was described by Tirimanna and Wickremasinghe [178]. Monohydroxyxanthophylls and carotenes could be separated on silica gel and polyhydroxyxanthophylls and epoxides on infusorial earth plates impregnated with vegetable oil. Nine of the fourteen carotenoids found could be identified.

Two HPLC methods for the separation of non-phenolic pigments in tea have been published. Kitada *et al.* [179] described various methods for the determination of several non-phenolic tea constituents (L-ascorbic acid, tocopherol, carotene and chlorophyll) in Japanese green, oolong and black tea by HPLC. Carotenes and chlorophylls were separated on Zorbax-ODS and detected with a UV–VIS detector. Sencha (Japanese green tea) contained higher amounts of carotenoids and chlorophyll than oolong and black tea. These compounds were not detected in tea brews prepared by the usual method. Recently, Taylor and McDowell [180] published a method for the rapid classification of the non-phenolic pigments of tea leaf. Fresh green tea leaf was extracted with methanol as described [181]. Separation was performed on a Hypersil-ODS column (5 μm, 250 × 4.6 mm I.D.) with gradient elution using acetonitrile–water (90:10) as solvent A and ethyl acetate as solvent B (1.5 ml/min, 0–20 min 100% A to 50% B, 20–30 min 50% B). The HPLC

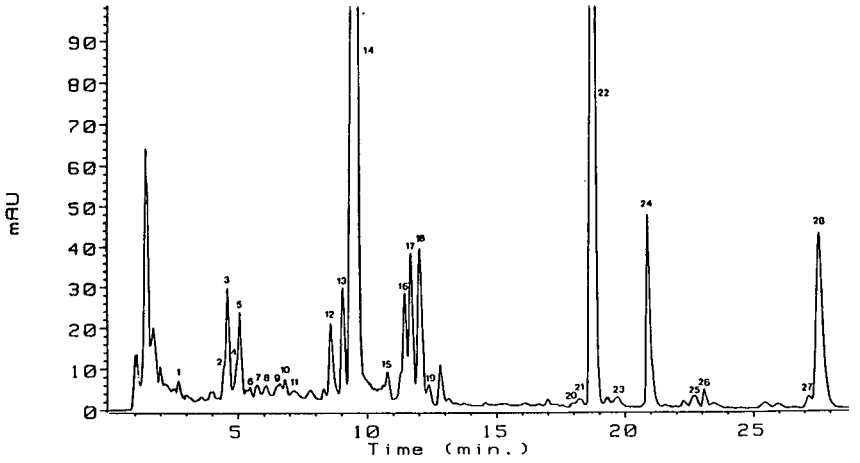

Fig. 13. HPLC elution profile of carotenoids and chlorophylls prepared from fresh leaf extracts of tea monitored at 450 nm. Identification of the main peaks: 3 and 5 = neochrome and flavoxanthin; 12 and 13 = luteoxanthin and flavoxanthin; 14 = lutein; 16 and 17 = mono isomers of lutein or lutein epoxide; 18 = isomer of lutein or lutein epoxide; 22 = chlorophyll *b*; 24 = chlorophyll *a*; 28 = β-carotene. From ref. 180, with permission.

trace recorded at 450 nm is shown in Fig. 13. Detection was carried out by DAD. The spectroscopic data of all 28 pigments obtained were given.

2.4.2. Amino acids

The following amino acids have been identified in tea leaves by PC or liquid chromatography [5]: aspartic acid, glutamic acid, glycine, serine, glutamine, tyrosine, threonine, alanine, valine, leucine, isoleucine, phenylalanine, lysine, arginine, histidine, tryptophan, aspargine and proline. In addition to these usual amino acids, tea contains a unique substance called theanine (5-N-ethylglutamine), which usually accounts for more than 50% of the free amino acid fraction of tea leaf and about 1% of the total dry weight. Fig. 14 shows the structure of theanine. Theanine is not found in tea proteins [182], but takes part in the biosynthesis of polyphenols. Correlations between content of amino acids and theanine and tea quality are discussed controversially in the literature. On one hand, green tea quality should depend to a large extent on amino acid level, especially that of theanine [8], and the highest quality black teas were found to possess the lowest amount of theanine [183], whereas on the other hand a high content of free amino acids is said not to be neither characteristic of green or black tea and no correlation between theanine and quality could be found [182]. Many of the amino acids in tea are involved in aroma formation. During black tea manufacture aldehydes are produced by flavanol quinone oxidation and through Strecker degradation of amino acids. Co and Sanderson [184] confirmed this mechanism in detail using a model tea fermentation system with radiotracer techniques and headspace GLC.

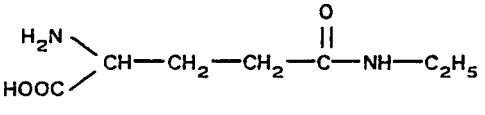

Fig. 14. Structure of theanine.

Early investigations on amino acids in tea using 2-D PC and the ninhydrin method partly after sample clean-up through a cation-exchange column were carried out by several groups [185–188]. They intended to examine the changes undergone by free amino acids, either as individual compounds or in total, during each processing step in the manufacture of black tea. In 1978 Chakraborty *et al.* [189] used this method to determine free amino acids in different parts of tea shoots and their effect on quality.

Theanine, aspartic and glutamic acid in green tea have been analysed by means of capillary tube isotachophoresis; however, these compounds could not be separated in a single run [190]. Especially for

theanine a GLC method after conversion into its TMS derivative was reported in 1974 [191]. The mass spectra were also discussed, but no method for tea sample preparation was given. Another very time-consuming method for determination of theanine in tea used a combination of TLC (4 h for one run!) and densitometry after a complex sample clean-up procedure [183,192]: extraction of tea with hot water, precipitation of polyphenolic and coloured substances with lead acetate, centrifugation, removal of excess lead by passing hydrogen sulphide through the solution and filtration.

Neumann and Montag [193] developed a method for the separation and determination of theanine in an automatic amino acid analyser together with twenty other amino acids. After purification of the aqueous tea extract by means of a strongly acidic cation exchanger, amino acids were separated with a special elution buffer system in about 160 min using ninhydrin reagent for detection (see Fig. 15). This method can be also employed for the separation of amino acids after hydrolysis.

Free amino acids and S-methylmethionine (anti-ulcer agent) in green tea extracts were simultaneously determined with an HPLC–amino acid analyzer and lithium citrate buffers which could also separate theanine and γ-aminobutyric acid from others. Analysis was carried out on a high-resolution column within 180 min. Conventional columns and sodium citrate buffers did not succeed in separating the amino acids satisfactorily [194]. Amino

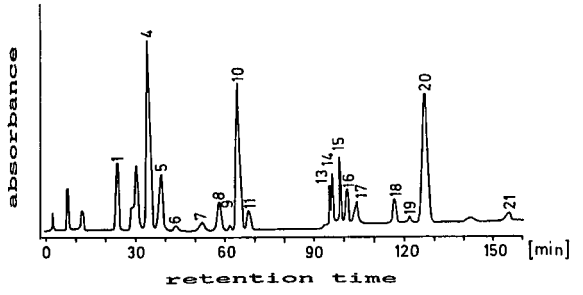

Fig. 15. HPLC separation of amino acids from a Taiwan oolong tea extract. 1 = Asparagic acid; 2 = threonine; 3 = serine; 4 = theanine; 5 = glutamic acid; 6 = proline; 7 = glycine; 8 = alanine; 9 = cysteine; 10 = homocitrullin (internal standard); 11 = valine; 12 = methionine; 13 = isoleucine; 14 = leucine; 15 = 4-aminobutyric acid; 16 = tyrosine; 17 = phenylalanine; 18 = lysine; 19 = histidine; 20 = NH_3; 21 = arginine. From ref. 193, with permission.

acid analysers are still applied [67,194,195], but have mostly been replaced with other more convenient methods such as HPLC with fluorescence detection or special RP-HPLC techniques [196–198].

After precolumn derivatization with dansyl chloride, sensitive determination of amino acids in green tea on a LiChrosorb RP-18 column was possible in about 32 min with a linear gradient of acetonitrile [198]. Zhu [196] determined theanine by HPLC with a RP-alkylphenyl C_{18} column, aqueous phosphoric acid as the mobile phase and detection at 240 nm. Analysis of amino acids by HPLC with fluorescence detection has become more common in the last few years. Hirose and Tamada [199] and Chang *et al.* [200] separated amino acids on ion-exchange columns, monitoring the fluorescence after reaction with *o*-phthalaldehyde in the presence of β-mercaptoethanol. The fluorimetric detection involved excitation at 350 nm and emission at 450 nm [200]. Other workers chose slightly different wavelength pairs: 338/425 [85], 360/500 [66], 340/455 [201], 335/450 [202] or 365/435 nm [203] for theanine alone and RP columns [66,201–203]. Using this sensitive method, a set of up to eighteen amino acids could be determined in about 90 min [85].

2.4.3. Metal speciation analysis

Research by chromatographic means has also been carried out in the field of metal speciation. The reason for these activities is the suggestion that tea polyphenols lower the absorption of Fe, Zn and Ca in the gut [204]. Disler *et al.* [205] showed in animal studies (rats) that the tannins in the tea are responsible for inhibiting the absorption of food Fe and medicinal Fe, probably by forming non-absorbable complexes with the Fe within the intestinal lumen. Brown *et al.* [206] also used rats for an animal study, and stated that beverages (coffee, black tea, cocoa and bush tea) reduced the bioavailability of Fe (whole-body retention of ^{59}Fe) by 31–45%. Individual polyphenols (EC and chlorogenic, gallic, caffeic and tannic acids) were less inhibitory than the beverages, reducing Fe bioavailability by 2–30%, while caffeic acid and tannic acid were the most inhibitory. Other workers (*e.g.*, ref. 207) stated that the addition of 100 mg of ascorbic acid or 200 g of milk completely counteracted the inhibitory effect of tea on Fe absorption. As Fe is one of the elements with a worldwide marginal status

[208], *in vivo* and *in vitro* studies have been undertaken. The binding of Cu, Zn and Fe to organic complexing compounds, tea and other beverages has been investigated [209–211]. The organically complexed metals were extracted using ethyl acetate and than analysed by RP-HPLC with UV–VIS detection and other more specific devices. The total amount of individual metals and their distribution in tea were determined by coupling HPLC and AAS. The results showed that polyphenolic compounds —especially the flavonoids— are important for the binding of the metals.

A radio-thin-layer method has also been used to detect the binding sites of Fe and Ni in tea [211]. To one tea sample ^{63}Ni or ^{59}Fe was added, followed by extraction with ethyl acetate and analysis by TLC on silica gel [with ethyl acetate or ethyl acetate–methyl ethyl ketone–formic acid–water (50:30:10:10)] or on RP-18 [methanol–water (70:30)], with radiometric detection. It turned out that the added metal ions are bound to the same compounds as Fe and Ni naturally occurring in tea and that strongly polar phenols play a major role in binding of Ni and Fe. The chromatographic behaviour of flavonoid standards and of flavonoids isolated from tea was analysed by HPLC (with electrochemical detection) and AAS. The metals (such as Cu, Fe and Al) are complexed by flavonoids containing vicinal OH bonds [212].

The high contents of aluminium in tea are also of interest. The level in tea made from twelve commercial brands was 3.9 mg/l (range 2.7–4.9 mg/l) [213], or from 2.2 to 4.5 mg/l (thirteen different tea infusions) as determined by AAS [214]. From an animal study [215] it was concluded that the Al in tea was very poorly absorbed but that tea, either in the form of an infusion or as tea leaves, had an adverse effect on Fe status.

2.4.4. Vitamins

Black tea contains only small amounts of some vitamins [8]. As the nutritional benefits of tea are not mainly due to vitamin contents, only a few methods have been published. The relatively high amounts of ascorbic acid in fresh tea leaves diminishes during fermentation. However, some determinations of vitamins by means of chromatographic methods have been published. In most instances green teas were analysed. Liang *et al.* [85] used HPLC for the detection of ascorbic acid. They found 2 mg/g in two green tea samples.

Tsushida and co-workers [216,217] also detected ascorbic acid in green tea by HPLC after extraction with 2% metaphosphoric acid by steeping for 10 min. Dehydroascorbic acid in the extract was chromatographed after reduction with H_2S. A modification of the indophenol titration method has been published [218]. Interfering colours could be removed by contacting with bovine skin powder. Ohtsuki *et al.* [194] described the determination of S-methylmethionine, which they called vitamin U, together with free amino acids (see Section 2.4.2) on a high-resolution column, MCI Gel CK-10U (150 mm × 4.6 mm I.D.). S-Methylmethionine was identified as dimethyl sulphide in the column eluates by GLC with flame photometric detection. Kitada *et al.* [179] used HPLC with an electrochemical detection system for the determination of ascorbic acid and HPLC with fluorescence detection for tocopherol in different teas. All the compounds examined were much more common in sencha than semi-fermented (oolong) and fermented (black) teas. The average contents of ascorbic acid and α-tocopherol in sencha were 167 and 13.5 mg per 100 g, respectively.

The riboflavin contents of green tea have been determined by Anan *et al.* [219] using HPLC. Tea was extracted with dilute HCl in boiling water for 30 min. The extract was mixed with an enzyme preparation. The HPLC system consisted of an ODS column and water–acetonitrile–acetic acid (88.5:11:0.5) as the mobile phase. A fluorescence detection system (excitation at 360 nm and emission at 500 nm) was used.

3. CONCLUSIONS AND FURTHER RESEARCH NEEDS

One of the major challenges in tea analysis is the improvement of our knowledge of the thearubigins, which is only possible by combining chromatographic and spectroscopic analysis with a suitable clean-up. Another problem, which is less on the analytical side, is to set up a suitable concept for volatile flavour compounds. In the field of metal speciation and bioavailability there is need for studies in which both the *in vivo* and the *in vitro* aspects are covered. Moreover, depending on the different composition and contents of polyphenolic com-

pounds, the effect of different teas on bioavailability needs to be checked.

Interdisciplinary research (chemical, biochemical and medical) is also needed for the improvement of our knowledge of the positive health effects of tea polyphenols. A chemical measure for tea quality has not yet been set up.

4. ABBREVIATIONS

Ac	Acetyl
AAS	Atomic absorption spectrometry
BSA	Bistrimethylsilylacetamide
CouQA	*p*-Coumaroylquinic acid
CQA	Caffeoylquinic acid
2-D	Two-dimensional
DAD	(Photo)diode-array detection
DMF	Dimethylformamide
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
FI	Flavour index
GLC	Gas–liquid chromatography
HPLC	High-performance liquid chromatography
IBMK	Isobutyl methyl ketone
IR	Infrared spectrometry
IUPAC	International Union of Pure and Applied Chemistry
MS	Mass spectrometry
NIR	Near-infrared spectrometry
NMR	Nuclear magnetic resonance spectrometry
PC	Paper chromatography
PVPP	Polyvinylpyrrolidone
RP	Reversed-phase
SDE	Simultaneous distillation and extraction
SEC	Size-exclusion chromatography
SPE	Solid-phase extraction
THF	Tetrahydrofuran
TF	Theaflavin
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TR	Thearubigin
UV	Ultraviolet
VIS	Visible

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Review

Analysis of organic micropollutants in the lipid fraction of foodstuffs

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ABSTRACT

An overview is given of current techniques for the analysis of organic micropollutants that accumulate in the fatty fraction of foodstuffs, such as pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polychlorinated dibenzodioxins and polychlorinated dibenzofurans. Isolation and clean-up are considered to be of great importance in the field of residue analysis. In general, problems are related to the low levels of the individual compounds at which they usually occur and the complexity of extraction and clean-up procedures for isolating and separating analytes from matrix components and other contaminants. Therefore, special attention is focused on sample pretreatment and on coupled chromatographic techniques, showing developments towards multi-residue methods, miniaturization and automation of analytical procedures. Coupling of chromatographic techniques with spectroscopic techniques is also considered as an important tool for identification and confirmation purposes.

CONTENTS

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1. INTRODUCTION

In the last few decades, considerable effort has been put into the development of analytical schemes for the determination of persistent halogenated hydrocarbons and pesticides in foodstuffs. The production and use of certain organic compounds in agriculture and the unintended formation of certain contaminants during chemical and combustion processes have led to a world-wide occurrence of these compounds in the biosphere. One result is that some foodstuffs may become contaminated by trace amounts of these compounds. Some groups of these compounds exhibit a high degree of persistence and predominantly accumulate in the lipid fractions of the human food chain, by which food has become a major route of exposure for humans.

In order to assess the daily exposure to organic contaminants in food by the general population, several field studies have been performed on the occurrence of polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), polychlorobiphenyls (PCBs) and, more recently, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in food [1–3]. Problems in chemical analysis are usually related to the low levels of individual compounds and the complexity of extraction and clean-up procedures to isolate the analytes from the food components, caused by the presence of major and minor components (matrix, other contaminants) which potentially disturb reliable identification and quantification. Several solutions have been proposed to decrease limits of detection (LOD) to the appropriate levels at which the analytes occur and to eliminate major sources of interference such as lipids, waxes and animal sterols in tissue extracts and carotenes in extracts from vegetable materials [4].

In order to identify and quantify residues of the different classes of organic compounds, well defined schemes have been established for the analysis of individual components or specific groups of compounds. To ensure and improve the reliability and comparability of analytical work in this field, these analytical schemes are well documented in reference texts, such as the *Pesticide Analytical Manual* (FDA) [5], the *Manual for Analytical Quality Control* (EPA) [6], the *Guide to Chemicals in Crop Protection* (1982) [7], *The Pesticide Manual* (UK) [8], *Analytical Methods for Residues of Pesticides* (Netherlands) [9], *Manual of Pesticide Residue Analysis* [10], *Official Methods of Analysis of the AOAC* [11] and *The Agrochemicals Handbook* [12]. Current methods for extraction, clean-up and group separation techniques in organochlorine trace analysis have been recently reviewed by the IUPAC Commission on Microchemical Techniques and Trace Analysis [4]. Further, several national and international frameworks (CEN, EC–BCR, WHO, FAO) frequently evaluate the analytical performance of laboratories performing analyses of specific analyte–matrix combinations by means of inter-laboratory comparison studies. This all may have led to a high degree of comparability of procedures applied in the analysis of foodstuffs and an acceptable level of reliability of the analytical results obtained.

In the last few years, several trends can be observed in the trace analysis of organic compounds. First, a growing tendency towards multi-residue methods for the simultaneous identification and quantification of several groups of compounds exhibiting similar environmental and/or toxic properties, such as pesticides, PCBs, PCDDs and PCDFs, can be observed. In this regard, developments usually involve adapting and modifying conventional techniques. Second, coupling of chromatographic and detection techniques, either off-line or on-line, miniaturization and automation of both sample pretreatment and analytical procedures have become of growing interest in the organic analytical field. Most of this work is primarily directed to have access to less time-consuming, miniaturized and automated procedures, providing useful tools for less expensive routine analyses in field studies and regulatory practice. Finally, other contaminants which pose a potential hazard after environmental and human exposure have become of interest. For some of

these compounds (*e.g.*, planar and mono-*ortho*-PCBs, polar pesticides, di- and tetrachlorobenzyltoluenes), a more profound development seemed to be necessary, as modifying available master schemes did not provide the necessary sensitivity and selectivity to determine these compounds at the levels at which they usually occur.

This paper critically reviews recently developed methods for the isolation/extraction and subsequent clean-up and determination of persistent halogenated hydrocarbons and pesticides in the lipid fractions of foodstuffs. Papers were selected by means of a literature search covering the period from 1985 until December 1991; for the historical perspective older references have also been included. The search was restricted to chromatographic techniques for the analysis of the following classes of compounds: pesticides (*e.g.*, OCPs), PCBs (including planar and mono-*ortho*-substituted congeners), polycyclic aromatic hydrocarbons (PAHs), PCDDs and PCDFs. Other reviews have been consulted to offer a broader view on current trends in the analytical field. Table 1 gives an overview of the analytical techniques currently employed by routine laboratories for the determination of various contaminants in fatty foods.

2. SAMPLE PRETREATMENT

Field studies dealing with food contaminants are usually performed either on total diet samples or on individual food products. Wells [4] reviewed current methods for the isolation of trace organics from several types of matrices.

In dietary intake studies [13], collected samples usually refer to pooled samples of food products (meat, fruit, vegetables, drinks, etc.) that an individual person has consumed during one day (24 h). In most studies, collected samples are initially freeze-dried to remove the water content and subsequently blended [14] in the presence of an organic solvent mixture such as acetone–pentane (1:1, v/v) or Soxhlet extracted [15] with organic solvents such as pentane or hexane to isolate the lipid fraction of the sample.

Methods for the isolation of lipid fractions from individual food products depend on the type of sample. Butter, fats and oils are generally assumed to be homogeneous, and normally do not require extensive extraction procedures. Aliquots of such samples can be dissolved in *n*-hexane or light petroleum to the desired concentration.

Meat products having a lipid content of *ca.* 10 wt.% or lower are initially blended and homogenized. Next, a representative test sample is ground with anhydrous sodium sulphate, until a free-flowing powder is obtained. This mixture can then be extracted by using either blending techniques (see earlier), a cold column extraction technique (elution of a column packed with a dried mixture with an organic solvent or solvent mixture) [16–18] or a Soxhlet extraction technique [15].

Milk can either be freeze-dried or chemically dried with anhydrous sodium sulphate, followed by Soxhlet extraction with organic solvent, or subjected to a liquid–liquid extraction procedure consisting of mixing with sodium oxalate and ethanol or methanol, followed by (repeated) extraction steps with a combination of organic solvents such as acetone–pentane [9] or diethyl ether–light petroleum [11].

Vegetable materials usually have a high water content and will dehydrate prior to analysis unless extracted immediately after sampling. Vegetables are therefore crushed, chopped and gently dried at 40–50°C prior to storage and analysis. Isolation procedures include grinding with coarse sea sand, blending, mixing with a more polar solvent (acetone) and subsequent partitioning with dichloromethane or hexane (applicable for matrices with a high sugar content) [19] or mixing with a more polar solvent (acetone) followed by either shaking or Soxhlet extraction [4].

The methods described above represent current techniques for isolation and are still widely in use at laboratories performing trace analyses of organic contaminants in foodstuffs. These methods have frequently served as the starting point for the variety of clean-up and separation methods described in the following sections. More recently, other methods for isolation/extraction, *e.g.*, solid-phase extraction, liquid–liquid extraction and supercritical fluid extraction, have been introduced. A brief description of the latest developments and a short evaluation of their applicability are presented. Sample purification schemes serve two purposes: removal of gross levels of co-extractants and separation of the organochlorine residues into groups, based on

TABLE I

OVERVIEW OF ANALYTICAL TECHNIQUES FOR DIFFERENT CLASSES OF ORGANIC MICROPOLLUTANTS CURRENTLY IN USE IN ROUTINE LABORATORIES

Class of compounds	Sample pretreatment techniques		Analysis	References
	Extraction	Clean-up		
PAHs	Saponification Solvent extraction Dimethylformamide–water partitioning Caffeine complexation	Adsorption chromatography on silica Adsorption chromatography on alumina Adsorption chromatography on XAD-2 Molecular partitioning on Sephadex LC-20	HPLC–fluorescence/UV GC–FID–MS	1,4,93,94, 130
PCDDs, PCDFs (planar PCBs)	Solid-phase (column) extraction Solvent extraction Soxhlet extraction Liquid–liquid partitioning	Adsorption chromatography on silica Adsorption chromatography on carbon Adsorption chromatography on alumina Adsorption chromatography on Florisil Gel permeation chromatography	GC–MS	2,4,58–61, 68,131
PCBs (planar)	Saponification Solvent extraction Liquid–liquid partitioning Soxhlet extraction	Adsorption chromatography on silica Adsorption chromatography on carbon Adsorption chromatography on alumina Liquid–liquid partitioning Treatment with sulphuric acid Gel permeation chromatography HPLC–Hypercarb column (PGC)	GC–ECD GC–MS	24,64,66, 68–69,81,82
PCBs (planar and mono-*ortho*)	Saponification Solvent extraction Liquid–liquid partitioning Soxhlet extraction	Treatment with sulphuric acid Carbon on silica (LC) Gel permeation chromatography HPLC fractionation–PYE column	GC–ECD, GC–MS GC–GC–ECD	24,63,83,84
PCBs	Solvent extraction Liquid–liquid partitioning Soxhlet extraction Saponification Acid hydrolysis	Liquid–liquid partitioning Adsorption chromatography on silica Adsorption chromatography on Florisil Adsorption chromatography on alumina Gel permeation chromatography Adsorption chromatography on carbon HPLC (silica)	GC–ECD GC–NPD/FDP GC–MS (confirmation)	4,24,132–136
Pesticides	Solvent extraction. Liquid–liquid partitioning	Adsorption chromatography on silica Adsorption chromatography on Florisil Adsorption chromatography on alumina Gel permeation chromatography Sweep co-distillation	GC–ECD GC–NPD/FPD GC–MS (confirmation)	4–12,26,34– 49, 82,83

their solid–liquid adsorption characteristics [4]. In the 1970s and 1980s, a number of useful conventional treatments for the removal of interfering co-extractants and organic macromolecules were introduced. However, some of these methods, *e.g.*. saponification with ethanolic potassium hydroxide for specific analysis of PCBs [20,21] and treatment with sulphuric acid (either by shaking or by using acid-impregnated silica) [22,23], are known to lead to a loss of other analytes such as chlorinated pesticides, an observation recently pointed out again by Wells [4] and De Voogt *et al.* [24]. As the scope of this review is directed towards advances in multi-residue analysis, only the less destructive methods will be described, *e.g.*, methods based on the use of activated carbon, magnesium silicate (Florisil), alumina and/or silica, and those implementing high-performance liquid chromatographic (HPLC) techniques and gel permeation chromatography (GPC). Recent reviews on the application of these techniques have been written by Erickson [25], Wells [4] and De Voogt *et al.* [24].

2.1. Liquid–liquid extraction

Liquid–liquid extraction is a well established technique used for the isolation of organic micropollutants from food samples. Isolation and clean-up techniques applied in the determination of pesticides in fatty foods were reviewed recently by Walters [26].

Typically, homogenized, representative subsamples are extracted once, or several times, with a water-immiscible solvent mixture such as hexane–acetone or light petroleum–diethyl ether. The analytes of interest, together with a wide variety of other lipophilic compounds, and the total lipid fraction are extracted in this way. After drying by filtration over anhydrous sodium sulphate, the crude extract is evaporated to dryness. After this procedure, the residue, consisting of the lipid fraction of the foodstuff, can be kept in a refrigerator or a freezer prior to further analysis. As this procedure isolates the fat matrix and the fat-soluble residues, an extensive clean-up and fractionation will be involved in further analysis. More selective isolation methods include liquid–liquid partitioning steps. When the foodstuff is of a solid nature, a (sub)sample is melted, macerated or blended with an aliquot of an apolar solvent (mixture) or depending on the texture, salt and/or sand is added before the (multiple) extraction with an apolar solvent (mixture) takes place [9]. Subsequently, solvent partitioning can be carried out by adding acetonitrile to the extract. The compounds of interest dissolve in the acetonitrile layer, while the fat remains mainly in the hexane or light petroleum layer. Because of the presence of residual fat a further clean-up of the acetonitrile extract is still necessary in most instances. The method can be applied to virtually all solid foodstuffs of animal and vegetable nature.

Prapamontol and Stevenson [27] developed a single-step extraction method for milk with ethyl acetate–acetone–methanol (2:4:4) by ultrasonification. Owing to the polar nature of this solvent mixture compared with conventionally used solvent, the amount of co-extracted fat was reduced significantly. This resulted in a considerable simplification of the subsequent sample clean-up process. The eleven organochlorine pesticides tested could be recovered quantitatively owing to the breaking down of the milk fat globules that can otherwise trap fat-soluble compounds.

A technique that is often used for the isolation of contaminants that are difficult to extract with conventional liquid–liquid extraction is Soxhlet extraction. In this way, continuous extraction of a sample with an appropriate solvent mixture, at elevated temperatures, if necessary for several days, can be performed without the need for much attention.

Huckins [28] described the use of semi-permeable membrane bags during Soxhlet extraction for separating the component fractions from the lipid matrix of foodstuffs. During extraction, components such as PCBs, PAHs, PCDDs and PCDFs are allowed to pass through the semi-permeable membrane of the polyethylene bag, while the lipid matrix is retained in the bag. An extraction time of only a few hours instead of the normal duration of 10–20 h in the original operation with toluene was sufficient for the quantitative recovery of all components of interest. Zebühr *et al.* [29] recently introduced a multi-residue procedure, including this isolation/extraction method followed by an automated HPLC clean-up method, for the analysis of residues of PCBs (including planar and mono-*ortho*-PCBs), PAHs, PCDDs and PCDFs.

Recently, automated equipment was introduced

for automated sequential trace enrichment of dialysates (ASTED); some of the applications published so far involve the determination of veterinary drugs and food additives in foodstuffs [30,31].

Recent developments in liquid–liquid extraction show a trend towards smaller sample sizes, resulting in savings on solvents and other chemicals, and also resulting in faster clean-up procedures [32]. The state of the art in the field of extraction methodology for pesticides was reviewed by Steinwandter [33]. However, it should be noted that a reduction in the amount of sample handled can only be achieved if the analytical method is sensitive enough to detect the reduced amount of the analytes. In some instances the efficiency of the analytical procedure can be enhanced by the use of on-line coupled techniques which totally transfer a small amount of the sample to the chromatographic system. Examples of such techniques are described in Section 3.

2.2. Adsorption chromatography

Adsorption chromatography has been used for over 35 years for the clean-up and fractionation of food sample extracts. Commonly used sorbents include magnesia, Florisil, silica and alumina; for some applications these sorbents have also been modified with, *e.g.*, silver nitrate or sulphuric acid. These materials show a polar behaviour, thus retaining the lipid fraction on elution with organic solvents of low polarity. This means that these sorbents are only suitable for apolar analytes, because more polar analytes will co-elute with the lipid fraction.

In earlier work in the USA and Canada, much effort was put into the development of methods involving magnesia or the synthetic magnesium silicate Florisil [34–36]. On the basis of this methodology numerous multi-residue applications were developed over the years [37,38]. It is interesting that these methods, developed more than 30 years ago, are still included in the official AOAC and FDA manuals [5,11].

Method development in Europe was focused more on clean-up procedures involving alumina and silica sorbents. For the separation of the fat from analytes of interest alumina has been extensively studied [39–42]. For the fractionation of the analytes, once the fat has been removed, either by alumina chromatography or another technique such as GPC or liquid–liquid partition, to a certain extent silica can be used for further clean-up [43–49]. These methods usually involve a liquid–liquid extraction technique, isolating the fat. Subsequently, the fat is separated from the organochlorine compounds of interest by alumina column chromatography, and after this the separation of the PCBs from the organochlorine compounds, necessary for a reliable gas chromatographic quantification, can be achieved by column chromatography over silica. A relatively new application of this procedure was described by Fürst *et al.* [50,51] for the determination of tetrachlorobenzyltoluenes (TCBTs; Ugilec) in fish samples. The most critical step in these schemes is the alumina chromatography; in order to obtain an efficient separation of the lipid fraction from the relevant compounds, the alumina must be deactivated to an appropriate degree by the addition of water. If too much water is added, fat retention will be insufficient. On the other hand, if the water content is too low, the analytes will not elute from the column within a reasonable time. Owing to the poor batch-to-batch reproducibility of these sorbent materials, exact adjustment of the chromatographic conditions is tedious and the hygroscopic properties of the materials make the storage of a prepared amount troublesome.

Major drawbacks of the techniques described above is that they are laborious and difficult to automate, combined with high solvent consumption. Therefore, several workers have investigated the feasibility of miniaturization of the chromatographic systems involved [52,53]. Miniaturization can result in the use of solid-phase extraction cartridges instead of the conventional glass chromatographic columns. Commercially available polypropylene tubes, typically filled with 100–1000 mg of sorbent, are being used. A wide range of materials such as ion exchangers, silica and alumina and reversed-phase type chemically bonded silicas have been tested. For the separation of analytes from lipids, the polar sorbents, conventially used in adsorption chromatography, are being used almost exclusively. The more recently developed phases such as chemically bonded silicas and modified carbons have the potential for clean-up purposes at least in specific application areas.

Because the activity of the sorbent cannot be ad-

justed batchwise off-line, an on-line adjustment has to be carried out. The extraction/clean-up procedure is as follows: the sorbent is conditioned by washing with an appropriate solvent, an aliquot of the crude sample extract is brought on to the column, the column is washed to eliminate matrix interferences and finally the analytes of interest are eluted. Clean-up of the extract also takes place because part of the interferences remains immobile on the sorbent during the elution step. Clean-up of milk extracts for the analysis of PCBs and organochlorine pesticides by means of solid-phase extraction cartridges has been studied by several workers [27,54,55]. An additional advantage of the use of solid-phase extraction cartridges is that procedures can be automated by the use of modified LC autosamplers [56].

The basis of carbon chromatography was laid by Stalling and co-workers [18,57,58], who described the potential of activated carbon chromatography for the specific fractionation of planar aromatic molecules. The retention of solutes is based on the coplanarity of closely situated aromatic systems and is increased by electronegative substituents (chlorine, bromine, nitro) on the aromatic systems.

A major drawback of finely divided carbon as a packing material is the high back-pressure. Stalling *et al.* [57] described a method for dispersing finely divided carbon, Amoco PX-21, on the surface of shredded polyurethane foam, improving the recoveries and separation of strongly adsorbed planar compounds. Later, carbon dispersed on glass fibres was used to fractionate non-ionic chlorinated pesticides and polar PCBs from planar PCBs and PCDD/Fs [57]. Clean-up through a series of silica-based adsorbents is necessary before application of the activated-carbon adsorbents.

A modified version of this procedure was reported by Liem *et al.* [59] for determination of PCDD/Fs in eel and milk, and Beck and co-workers [60,61] also applied a slightly modified carbon adsorbent for the determination of PCDD/Fs and PCB 77 in human milk and tissue.

Miyata *et al.* [62] used active carbon on silica for the fractionation of polar PCBs from planar PCBs and PCDD/Fs with elution with toluene at 80°C for the analysis of Yusho oil and tissues of patients with Yusho disease. Mixing of activated carbon with silica [carbon AX-21–silica (1:1)] for use in low-pressure LC in the clean-up of fish samples was performed by Hong and Bush [63]. Stepwise elution with different eluents resulted in several fractions containing 2–4 *ortho*-substituted PCBs, mono- and non-*ortho*-PCBs and PCDD/Fs. Norén *et al.* [64] utilized a mixture of activated charcoal (SP-1) and Chromosorb W in a final step in sample preparation for the determination of planar PCBs in milk.

In several studies activated carbon was used without modification or mixing, often resulting in a lengthy clean-up (using several eluents and large elution volumes) and broad and tailing elution profiles as a result of the inhomogeneity of the active sites on the activated carbon [65–67]. To reduce the large volumes of different solvents, Liem *et al.* [68] introduced Carbosphere activated carbon, which has a high loading capacity for fat and a low affinity for lipids and which is used in a reflux unit. For the isolation of PCDD/Fs from extracts of milk only 40 ml of toluene is used in a back-reflux mode whereas others use 200 ml of solvent. The same method can be used for the determination of planar PCBs in milk and other food matrices (fish, meat, butter, cheese), in which the analytes are recovered after direct refluxing with toluene for 1.5 h [69].

2.3. HPLC sample clean-up

Adsorption chromatographic methods, using alumina, magnesium or silica-based material, as described in the previous section are laborious and automation is difficult. HPLC and solid-phase extraction techniques are more suitable for the development of automated techniques. HPLC has additional advantages because first, it has a high separation potential compared with SPE or classical column chromatography and second, the separation process can be followed directly by UV or refractive index (RI) detection. Gillespie and Walters [70] introduced a procedure using a semi-preparative silica HPLC column (250 × 9.2 mm I.D., packed with 6-μm porous spherical particles) to separate OCPs and PCBs from butter fat. Solutions containing 0.4 g/ml of fat in hexane were injected on to this column. With a mobile phase of dichloromethane–hexane (20:80) at a flow-rate of 4 ml/min, 300 mg of fat were well resolved from five different organochlorine pesticides together with the PCBs. The elution of these compounds, however, required

148 ml of mobile phase. The method was compared with the official AOAC method [11], which is based on Florisil column chromatography. LC was found to be superior with respect to the time of analysis and the separation efficiency.

Dolphin *et al.* [71] introduced LC column switching for the automated analysis of OCPs in milk extracts. A precolumn (50 × 2.1 mm I.D., packed with 5-μm Partisil) was used for the retention of the fat and for the separation of the more polar pesticides (*e.g.*, β-HCH, heptachlor epoxide and dieldrin), whereas an analytical column (150 × 3.1 mm I.D., 10-μm Partisil) resolved the early-eluting compounds (*e.g.*, HCB, DDT complex and α-HCH). For both columns the mobile phase was *n*-hexane. The pesticides were detected with an electron-capture detector coupled directly to the LC system. Fairly high limits of determination (0.1 ppm level) were reported. This procedure is less suitable for the PCBs because LC cannot provide an adequate separation of the individual congeners.

A similar procedure was used for sample pretreatment prior to gas chromatography (GC) for the determination of organochlorine pesticides and PCBs in human milk [55]. On-line LC–GC is less suitable for the OCPs as no group separation for this heterogeneous group of compounds is available by LC. Hence no simple heart-cutting technique will be available for these compounds. Therefore, an off-line LC procedure was developed in which both the PCBs and the OCPs can be handled in a single clean-up procedure. In this work a completely automated clean-up with an LC system involving column switching for the separation of OCPs and the PCB fraction in fat extracts prepared from human milk was described. With this procedure it was possible to obtain separated fractions of OCPs and PCBs prior to capillary GC–electron-capture detection (ECD) with a capacity of 20 samples per day.

The availability of automated HPLC is one of the factors that led to the development of gel permeation chromatography (size exclusion or GPC) as we know it today. GPC is a powerful preparative chromatographic clean-up procedure which can be used prior to HPLC and GC analysis [45,72,73]. The original applications used Bio-Beads SX2 and cyclohexane as eluent with a 270 × 20 mm I.D. column, allowing injections of up to 500 mg of fat. Nowadays, commercially available auto-preparative systems use Bio-Beads SX3 and toluene–ethyl acetate (1:3) as eluent with an increased loading capacity of up to 1–2 g of fat per injection.

GPC has several advantages over atmospheric pressure chromatography. First, the same column can be used for the clean-up of large series of samples. Second, the clean-up procedure itself can be monitored with a UV or RI detector. This leads to a technique that can easily be automated. Separation with GPC is based on molecular size rather than on boiling point and/or polarity, thus adding selectivity to the analytical procedure, as the smaller molecules show the highest retention. It is an ideal technique for the separation of macromolecules such as lipids and pigments from lower molecular mass organochlorine contaminants.

A drawback is the relatively low separation power of GPC in the lower molecular mass range, resulting in large eluate volumes and in the impossibility of performing fractionation. In addition, GPC seems to be less suitable for automated routine analyses of planar PCB congeners and the toxic PCDDs and PCDFs, as even the most sensitive GC–mass spectrometric (MS) procedures demand sample amounts of at least 5 g of fat or more. Increasing the loading capacity by enlarging the GPC column dimensions will inevitably result in a large throughput of solvents and adsorbents, introducing high costs and higher risks of contamination [24]. For these groups of compounds, activated carbon (see the previous section) and the use of the recently introduced 2-(1-pyrenyl)ethyldimethylsilylated silica column in HPLC [74] seem to be more appropriate. In the applications discussed in this section HPLC is used as a preparative technique. This facilitates the use of more exotic materials compared with analytical HPLC, because bad peak shapes are less a problem.

As noted in Section 2.2, carbon-based materials have a fair potential in the clean-up of PCBs and PCDD/Fs. A new development in carbon-based chromatographic materials is porous graphitic carbon (PGC). PGC is an amorphous glassy carbon containing micropores and mesopores. On a colloidal level it has a strong sponge-like structure, capable of withstanding considerable shearing forces, rendering it suitable for HPLC [75]. The surface area is about 150 m^2/g, the mean pore volume 2.0 cm^3/g and the particle porosity 70%. The advan-

tages of PGC over activated carbon are that it typically uses a single eluent instead of a step gradient and sharp peaks can be obtained, because of the homogeneous nature of active sites of PGC. Disadvantages are that PCDD/Fs have to be recovered by backflushing the column, giving broad peaks (200 ml of hexane, or a smaller volume of toluene) and that preliminary pretreatment of extracts is necessary, as PGC has a relatively low capacity for co-extracted organics, causing overloading of the columns [76]. Much research has been carried out on the properties of PGC itself [75–77] and in relation to silica and organic polymers as packing materials for HPLC [78–80]. With PGC, a non-polar adsorbent, so that the solute retention is based on the balance between the non-specific intermolecular interactions, polar solvents have lower elutropic strengths; dichloromethane and dimethylformamide are the strongest solvents. Commercial PGC columns are now available, Creaser and Haddad [76] were the first to use a Shandon Hypercarb column (7 μm, 50 × 4.7 mm I.D.) for the HPLC separation of pesticides, lower chlorinated PCBs, planar PCBs and PCDD/Fs using hexane as mobile phase (5 ml/min). An improved separation could be achieved with acetonitrile–water (80:20). A typical LC trace of PCBs and PCDD/Fs on Hypercarb is shown in Fig. 1.

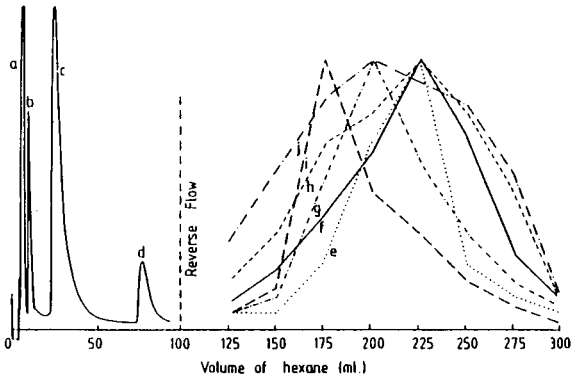

Fig. 1. Separation of PCBs and pesticides from PCDDs and PCDFs on porous graphitic carbon (50 × 4.7 mm I.D.; eluent, hexane, 5 ml/min). (a) PCBs (Aroclor 1254 + 1260) and pesticides; (b) 3,3′,5,5′-T_4CB and 3,4′,5-T_3CB; (c) 3,3′,4,4′-T_4CB and 3,4,4′,5-T_4CB; (d) 3,3′,4,4′,5,5′-H_6CB; (e) 1,2,3,4,6,7,8-H_7CDD and O_8CDD; (f) 1,2,3,7,8-P_5CDD, 1,2,3,7,8-P_5CDF, 1,3,7,8-T_4CDD, and 1,2,7,8-T_4CDD; (g) 1,2,3,4,7,8-H_6CDD; (h) 1,2,3,6,7,8-/1,2,3,7,8,9-H_6CDD and 1,2,3,4,8,9-H_6CDD; (i) 1,2,3,4,6,7,8-H_7CDD; (j) O_8CDF. 0–100 ml, UV detection (245 nm); 100–300 ml, GC–ECD analysis of discrete fractions. From Creaser and Al-Haddad [76].

Tuinstra *et al.* [81] demonstrated the use of the Hypercarb column for the determination of planar PCBs in horse fat. Samples were extracted and subsequently cleaned with GPC and alumina, before HPLC separation using cyclohexane–dichloromethane (1:1) (2 ml/min) as eluent for the first fraction (0–30 min) of OCPs and non-planar PCBs (Nos. 28, 52, 101, 105, 118, 138, 153 and 180) and switching to toluene for the second fraction (30–60 min) containing the planar PCBs (Nos. 77, 126 and 169). A large difference in the results between analysis with GC–ECD and GC–MS was found owing to interferences during ECD, which needs more clean-up.

Böhm *et al.* [82] also used HPLC fractionation with a Hypercarb column (10 × 4.7 mm I.D.) for the determination of planar PCBs in food using PCB 169 as internal standard, which may lead to a loss of relevant information as PCB 169 has been detected in a variety of foodstuffs [68] and in human milk [69]. GC–ECD was used in the analysis step instead of the more sensitive GC–high-resolution MS instrumentation.

Another new packing material with a potential similar to PGC for the separation of planar components is 2-(1-pyrenyl)ethyldimethylsilylated silica (available from Cosmosil as 5-PYE, 5 μm, 150 × 4.6 mm I.D.). PYE was found to be intermediate between silica and PGC with respect to the selectivity based on electronic and steric interactions with the fused-ring aromatic systems on the stationary phase, whereas carbon chromatography is based on charge-transfer interactions. The pyrenyl group on the PYE phase possesses fused aromatic systems with sixteen π-electrons, which may be regarded as a small part of a graphite surface and therefore it provides a much greater effect of a planar aromatic structure on retention than silica [80]. Advantages over carbon columns are the higher efficiency, less tailing elution profiles, as can be seen from Fig. 2, no irreversible adsorptions and a better batch-to-batch reproducibility.

Fig. 2 demonstrates the use of the PYE column for the isolation of mono-*ortho*- and planar PCBs from biological samples (fish and animal) as performed by Haglund *et al.* [74,83]. Hexane was used

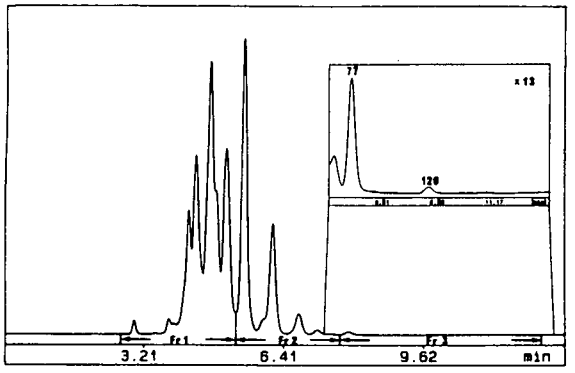

Fig. 2. HPLC showing the fractionation of Clophen A50 on a 150 × 4.6 mm I.D. PYE column. The numbered peaks correspond to 3,3',4,4'-T_4CB (IUPAC77) and 3,3',4,4',5-P_5CB (IUPAC 126). For chromatographic conditions, see Haglund *et al.* [74]. From Haglund *et al.* [74].

as the mobile phase, resulting in two fractions containing the bulk PCBs and mono-*ortho*-PCBs (Nos. 105, 118, 156, 157 and 159) in a small volume of 2.5 ml; planar PCBs were eluted in the backflush mode with a higher flow-rate (1.2 ml/min). Windows were defined with PCB 118 and 77, the first eluting congeners for mono-*ortho*- and planar PCBs, respectively. Retention in HPLC is reduced by matrix effects of non-hydrolysed lipids, which could be removed by GPC. To obtain optimum performance of the column it is necessary to remove the lipids almost completely. The same method was used for the determination of planar PCBs in fish [84].

2.4. Supercritical fluid extraction

Sample pretreatment with supercritical fluid extraction (SFE) is a relatively new technique in comparison with the other techniques discussed. The advantages of SFE over conventional liquid extraction methods are that it saves time and extraction solvents, it opens ways to more efficient extractions, selectivity is enhanced and it can easily be coupled to other chromatographic techniques. These advantages originate from the properties of supercritical fluids. First, the selectivity of extraction procedures can be tuned by the varying solvating power of the solvent as a function of its density, so that discrete fractions of analytes can be isolated from a sample by density and pressure programming. Second, rapid mass transfer during extraction is facilitated by the low viscosity and high solute diffusivities due to the liquid- and gas-like behaviour of supercritical fluids, thus improving the efficiency of the extraction process.

At present, carbon dioxide (CO_2) is most often used as the extraction solvent because of its moderate critical temperature (31°C) and pressure [73 atm (1 atm = 101.325 kPa)], it is non-flammable, non-toxic and relatively inexpensive and there are no waste problems. Modifiers can simply be added to adjust the solvating power of the fluid beyond the range that is accessible by density programming.

Until now, only few applications of SFE of fatty foods have been published. Generally, two different approaches can be distinguished in the application of SFE for sample pretreatment of fatty foods: using SFE to extract as many components and fat from the matrix as possible followed by an additional on- or off-line clean-up [85–90]; and selective extraction of only the components of interest by a proper choice of parameters [87,88,91]. An example of these two approaches was given by King [89], who studied the behaviour of porcine fat spiked with traces of DDT by varying the pressure. At low pressures up to 95 atm only a negligible yield of the lipid background was recovered. On increasing the pressure to 204 atm a finite yield of fat was observed combined with 75% recovery of DDT, and further extraction at 300 atm gave a significant amount of fat and some additional DDT. From these experiments it can be deduced that it should be possible to extract most of the DDT from lipid interferences if the extraction is performed at *ca.* 100 atm.

Hopper and King [90] used extreme extraction conditions (69 MPa, 80°C and 5 l/min of CO_2) to extract pesticides and co-extracting lipids from butter fat and peanut butter. Extraction was followed by an additional clean-up with GPC and Florisil. Good results were reported for the sample preparation by mixing the sample with an extraction enhancer (pelletized diatomaceous earth) to remove moisture and to prevent channelling during extraction.

Nam *et al.* [88] performed extractions of several pesticides from spiked dairy products, which were mixed with Florisil and sulphate, suspended on silanized glass-wool and homogenized, under conditions of 150 atm and 50°C for 30 min followed by clean-up with GPC. For thiophosphate and phen-

oxy ester herbicides good recoveries were found (70–95%); for triazines and carbamates the recoveries were lower (50–70%) owing to their polarity. Improvements were achieved by the use of a modifier (chloroform or methanol).

For the extraction of PAHs from spiked fish samples and 2,3,7,8-TCDD from spiked liver, higher pressures were necessary (170 atm and 50°C for 30 min); addition of a small amount of toluene to the extraction cell increased the extraction efficiency. The recoveries for TCDD were better than 70% at levels of 50 ppt–1 ppb (ppt = parts per 10^{12}; ppb = parts per 10^9).

Selective extraction procedures were performed by Nam *et al.* [88] using off-line SFE to extract chlorinated pesticides and PCBs from milk. The spiked samples (1–20 ppb) were mixed with Florisil and sulphate, suspended on silanized glass-wool and homogenized. Extraction was done at 160 atm and 50°C for 30 min and samples were trapped in hexane. The extraction efficiencies obtained with SFE were comparable to those obtained with liquid extractions.

Murphy and Richter [91] demonstrated the extraction of aldrin from soybean oil; selection of the optimum pressure was performed on the basis of recovery of the aldrin and co-extraction of lipids. A pressure just below that for the optimum recovery gave less matrix interferences. Good recoveries were found for all spiked matrices and were not improved by adding a modifier to the extraction cell.

SFE is a promising technique for the extraction of all kinds of matrices. Selective extraction procedures are preferred, where precise control of the extraction pressure is used to facilitate enrichment of the component from co-extractants from the matrix.

3. CHROMATOGRAPHIC TECHNIQUES

3.1. Liquid chromatographic techniques

3.1.1. Liquid chromatography

Today GC still appears to be the major analytical technique for residue analysis owing to its high separation power (capillary column), the availability of selective and sensitive detectors and, perhaps the most important factor, most of the laboratories involved in residue analysis are, for historical reasons, better equipped with GC systems. However, the application of HPLC is growing, especially for the analysis of pesticides that cannot be analysed directly by GC owing to poor volatility, high polarity and/or thermal instability of the compounds. Currently special reversed-phase (RP) columns have been introduced for the analysis of groups of compounds, *e.g.*, PAHs, carbamates, phenylurea herbicides and nitrophenols.

Brodsky and Ballschmiter [92] demonstrated the potential of HPLC as a confirmatory step in the isomer-specific determination of PCBs. They compared six different stationary phases of modified silica gel (Nucleosil $5C_{18}$ and 5CN, Hypersil ODS C_{18}, Sepralyte Diphenyl, Vydac 201 TP C_{18} polymer and R Sil HLDA C_{18}) for their separating properties by determining the retention indices of 87 PCB congeners in technical PCB mixtures. The highest selectivity was obtained with Nucleosil $5C_{18}$ with 75–90% methanol as eluent. Certain PCB congeners (*e.g.*, with IUPAC Nos. 132 and 153), usually difficult to separate in conventional GC, could be separated by using LC. It was concluded that LC can be used for confirmatory purposes.

Vaessen *et al.* [93,94] evaluated methods and associated problems observed in inter-laboratory comparison studies on the determination of PAHs in samples of coconut oil and green kale. As all the participating laboratories (fourteen) were considered to be experienced, results from this study represent the current state-of-the-art of PAH methodology in the field of food analysis. An overview is given of current techniques for the extraction, clean-up and analysis employed in PAH analysis. HPLC and glass capillary GC were the main techniques used for the separation and determination of PAHs. For the HPLC analysis of PAHs, Vydac columns provide the best answer to this analytical problem at present.

3.1.2. LC–LC coupling

An important feature of HPLC is the application of (pre)column switching, which offers the possibility of integrating sample preparation and clean-up in the chromatographic procedure. The use of multi-dimensional chromatographic procedures in LC also alleviates the major drawback of the technique *i.e.*, the lower separation power compared with capillary GC. The first papers on residue analysis of

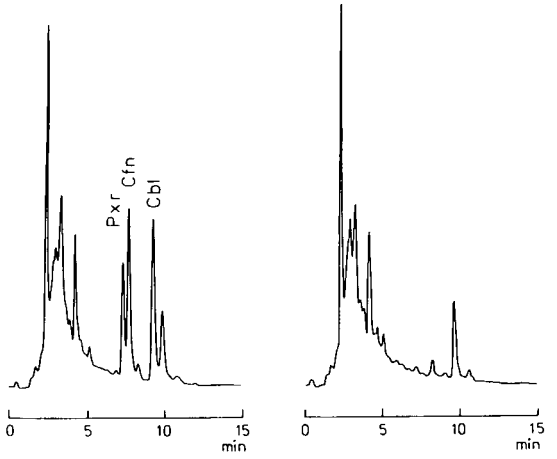

Fig. 3. Analysis of a 1-ml aliquot of total diet extract (5 g/ml). (a) Spiked with propoxur (Pxr, 5.5 μg/kg), carbofuran (Cfn, 6.4 μg/kg) and carbaryl (Cbl, 7.5 μg/kg). (b) Blank. LC column-switching technique according to Goewie and Hogendoorn [99].

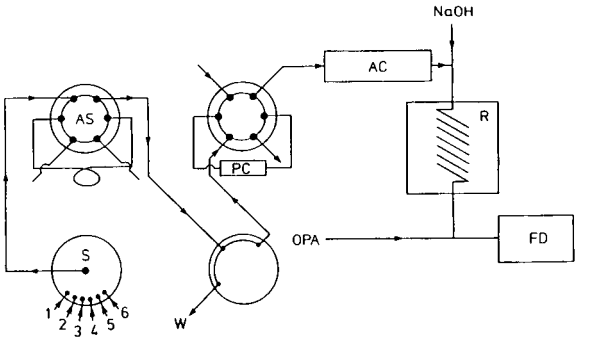

Fig. 4. Schematic diagram of the equipment for on-line precolumn switching HPLC analysis and postcolumn reaction used for the determination of N-methylcarbamate pesticides. S = Selector valve (low-pressure); AS = autosampler; PC = high-pressure switching valve with precolumn; W = high-pressure selector valve with "waste" line; AC = analytical column; NaOH = hydrolysis reagent; R = reactor coil; OPA = *o*-phthaldialdehyde, reagent mixture; FD = fluorescence detector. From Goewie and Hogendoorn [99].

pesticides with RP-LC column switching dealt with the preconcentration of analytes from aqueous samples, such as river water, soft drinks or serum [95–97]. For these applications precolumns of 2–10 mm × 2–3 mm I.D., packed with 5–10-μm C_{18} particles, were optimum with regard to loadability and performance. With this approach a manual extraction procedure is avoided. An obvious limitation is that the application field is limited to aqueous samples. Even with those samples a filtration step must always precede the on-line procedure in order to prevent clogging of the precolumn. As a consequence, the part of the analyte adsorbed on solid particles present in the sample must be analysed separately in order to obtain the total analyte content of the sample. Owing to their low separation power, these small precolumns are less suitable for clean-up purposes, as they cannot provide enough separation between analytes and sample interferences.

More recent studies using HPLC column switching for pesticide residue analysis have established that the use of longer (pre)columns in combination with off-line extraction/concentration results in a considerable selectivity enhancement [98].

An example of the use of precolumn clean-up of duplicate diet samples for N-methylcarbamates was given by Goewie and Hogendoorn [99]. The method was based on the improved HPLC method of Krause, extended with an automated column switching. Fig. 3 shows a typical chromatogram of the procedure and the experimental set-up is shown in Fig. 4.

3.1.3. LC–GC coupling

As stated before, capillary GC still remains the major analytical technique in residue analysis. A major drawback of capillary GC is that it has, contrary to LC, a low tolerance towards involatile and polar sample constituents, because these interferences cannot be removed effectively from a GC sys-

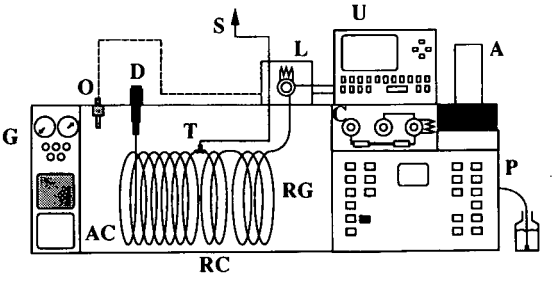

Fig. 5. Schematic presentation of the equipment used for LC–GC analysis. A = Autosampler; P = HPLC pump; C = LC column with switching valves; U = UV–VIS HPLC detector; G = gas chromatograph; L = loop-type interface; O = on-column interface: RG = retention gap; RC = retaining precolumn; AC = analytical column; S = solvent vapour unit; D = GC detector.

tem. For this reason, GC usually requires extensive clean-up procedures using solid–liquid chromatography. HPLC is far less critical in accepting dirty samples, and therefore coupling of HPLC with capillary GC seems to yield a very powerful combination. Especially the introduction of interfaces capable of transferring relatively large volumes of the LC eluate (typically 1 ml) greatly facilitated the development of LC–GC, as standard LC dimensions and flow-rates can be combined with capillary GC [100]. Equipment for LC–GC is commercially available now. Fig. 5 shows a schematic diagram of such an instrument. Maris *et al.* [101] reported on the use of a LC–GC interface for the enrichment of PCBs and pesticides from aqueous and sediment samples at the ppt level. Their system consisted of a micro-RP-LC column directly coupled to the capillary GC system through an interface consisting of an on-column injector and a retention gap.

Interfacing LC to GC can yield fast, reliable and automated analytical techniques. Most of the interfaces used today are based on concurrent or partially concurrent solvent evaporation. Concurrent solvent evaporation is a very powerful technique because it allows the injection of an, in principle, infinite volume of liquid into a capillary GC system. Many applications of LC–GC are based on the transfer of a heart-cut from a normal-phase liquid chromatogram, hence these techniques are extremely selective owing to the fact that the total chromatographic process is multi-dimensional.

On-line coupled LC–GC was reviewed extensively by Grob [100]. The technique is based on transferring a heart-cut from an LC column to a capillary GC system. PCBs can be determined in sediments with a relatively short sample pretreatment [99]. Grob *et al.* [102] reported an LC–GC method for the determination of PCBs in fish. In this study fat was well separated from the PCBs but the LC separation was insufficient to separate the OCPs from the PCB fraction. In these studies, however, resolution between OCPs and PCBs was not ideal because six OCPs co-eluted with the PCBs. Grob *et al.* [103] recently discussed the capacity of silica columns for retaining fat.

Barcorolo [104] used a modified ODS silica phase with isooctane as the mobile phase for the determination of organochlorine pesticides in fat-containing samples. Pesticides elute rapidly under these conditions whereas the fat is well retained by the stationary phase. The column was regenerated by rinsing with *n*-hexane.

One of the most important clean-up methods in residue analysis is gel permeation chromatography (GPC). Miniaturized GPC seems to be a logical candidate for LC–GC applications. The selectivity of this method lies in the separation of the analytes in one, relatively small, fraction from macromolecular interferences from the samples. Miniaturized GPC with a 0.32 mm I.D. fused-silica capillary column packed with 5-μm Rogel with a pore size of 5 nm has been shown to separate PCBs from triglycerides [105]. However, the application shown (10% of PCBs in oil) is far from the levels needed for practical purposes. A major problem in coupling GPC with GC is still the problem of tailing triglyceride peaks, destroying the capillary GC column. In a recent paper, Grob and Kalin [106] claimed that a large part of the peak tailing is caused by the LC injection valves, and therefore suggested putting the injection valves off-line to the main flow scheme of the LC–GC system.

Some developments in interfacing LC to GC can be expected from programmed-temperature vaporizer (PTV) split inlet [107–110]. With this method, a solvent purge technique is applied to preseparate volatile material, including the solvent, from high-boiling solutes in the injector. During the initial split-open period, solvent and low-boiling compounds are allowed to evaporate, while high-boiling components remain cold-trapped in the injection liner. Next, the splitter is closed and the inlet is rapidly heated, allowing the trapped components to enter the column. Grob [111] recently discussed the potential of PTV injectors for LC–GC interfacing.

3.2. Gas chromatographic techniques

The growing tendency towards multi-residue methods combining a high sample throughput with high levels of reproducibility and accuracy have led to improvements in column technology and knowledge concerning the retention behaviour of specific component groups (*e.g.*, PCBs, PCDD/Fs). In addition, improvements can be observed in multi-dimensional GC, LC–GC and SFE–GC, showing their potential for application in food analysis. In this section, recent developments are described.

In residue analysis, specific GC detection techniques such as electron-capture detection (ECD), nitrogen–phosphorus or thermionic detection (NPD) and flame photometric detection (FPD) are used. Numerous applications can be found for the detection of PCBs and the large group of nitrogen- and/or phosphorus-containing pesticides, including triazines and organophosphorus esters, and sulphur-containing compounds. Good overviews are given in the biannually published reviews in *Analytical Chemistry* [112,113]. The field of GC detection is more or less stable. The only noticeable recent development is the availability of a commercial instrument for atomic emission detection (AED), which is able to detect different specific elements including carbon, phosphorus, nitrogen and metals, such as tin and lead in organometallic compounds. A tendency towards the combined use of several different detection techniques to allow proper identification can be observed. The following sections refer only to recent developments in column technology in GC, the use of multi-dimensional GC and on-line SFE–GC techniques. Progress in the use of spectroscopic techniques is described in Section 4.

3.2.1. Gas chromatography

The inability of non-polar columns to separate all analytes of interest in a single GC run [114,115] and the thermal instability of highly polar stationary phases [115,116] has led several workers to the application of other and more stable stationary phases, *e.g.*, liquid crystal capillary columns [114,117,118], OH-terminated stationary phases [116] and columns coated with graphitized carbon black (GCB) [119,120]. Advances in column technology can be observed especially for the isomer-specific analysis of PCBs, PCDDs and PCDFs. The chemical and physical properties of pesticides are heterogeneous and therefore no real separation problems are encountered in this field.

Larsen *et al.* [115] recently tested commercially available columns for the analysis of non-*ortho*- and mono-*ortho*-PCB congeners by comparing the retentions of 140 PCB congeners in GC–ECD and GC–MS analyses on six narrow-bore fused-silica columns: SIL-5 (dimethyl), SIL-8 (5% diphenyl–dimethyl), SIL-19 (14% cyanopropylphenyl–1% vinyldimethyl), SIL-88 (biscyanopropylphenyl), HT-5 (1,2-dicarba-*closo*-dodecaboranedimethyl) and SIL-8–HT-5 (SIL-8 and HT-5 coupled in series). Of these, the HT-5 column offers the highest maximum temperature range allowing rapid analyses. It was concluded that no single GC column was able to separate all toxic PCBs from co-eluting congeners. Even the classical methylphenyl (5%)–polysiloxane phases for PCB analysis (*e.g.*, SE-54, DB-5, SIL-8), appeared to be an inferior choice for planar PCB analysis. The best choice for planar PCB analysis was the SIL-8–HT-5 combination, whereas the best overall performance (clear separation for 37 of 52 potentially toxic PCBs) was found for the non-polar dimethylsiloxane phase.

An alternative column for planar PCB analysis was recently introduced by Fischer and Ballschmiter [121,122]. They showed that, within a mixture of 176 PCBs, the planar PCB congeners can be clearly eluted last within the group of homologues by using a 50-m capillary coated with an 86% dimethyl–14% cyanopropylphenyl polysiloxane phase (*e.g.*, OV-1701, SB Octyl 50).

In the field of PCDD/F analysis, Schmid and Schlatter [116] compared the separation characteristics of glass capillaries coated with five different polysiloxanes, SP-2330 (10% methyl–90% 3-cyanopropyl), OV-240-OH (OH-terminated 67% methyl–33% 3-cyanopropyl), OV-225-OH (OH-terminated 50% methyl–25% phenyl–25% 3-cyanopropyl), OV-17-OH (OH-terminated 50% methyl–50% phenyl) and PS 247.5 (OH-terminated 100% methyl). The comparison showed a high coating efficiency with immobilized polysiloxane coatings, allowing the baseline resolution of all PCDD/F isomers with short columns. The OV-225-OH polysiloxane offered baseline separation of all congeners of interest and was also able to distinguish homologous groups and was therefore chosen as the stationary phase with the most suitable selectivity.

Ryan *et al.* [114] presented an extensive study on the GC–ECD separation of all 136 tetra- to octa-PCDD/Fs on nine stationary phases, including non-polar (DB-1, 100% methyl; DB-5, 5% phenyl), medium-polarity (DB-17 and OV-17, 50% phenyl–50% methyl; DB-210, trifluoropropyl), polar (DB-25, CPS-1, SP-2331, CP-Sil88, cyanopropyl) and others (SB-smectic, liquid crystalline). Except for certain pairs of PCDDs with 1,2,4-substitution, most of 136 PCDD/Fs can be readily separated by the use of a combination of two or more conven-

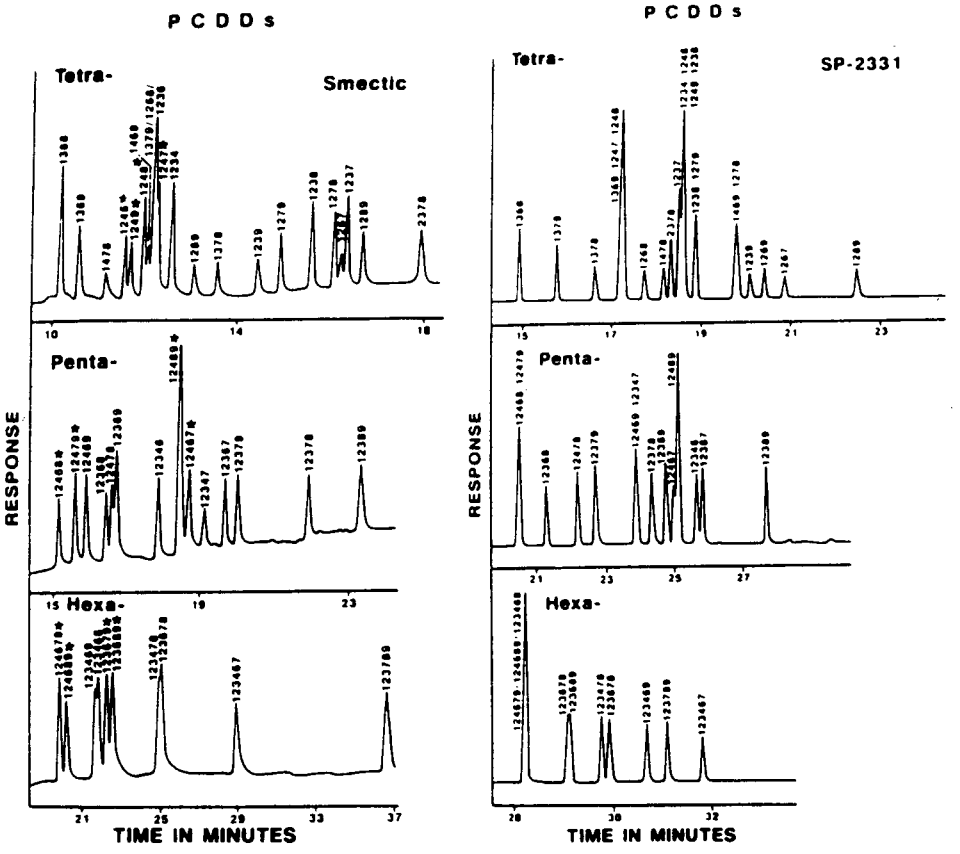

Fig. 6. GC–ECD trace of the separation of 22 T_4CDDs, 14 P_5CDDs and 10 H_6CDDs on an SP-2331 (left) and a liquid crystalline smectic (right) fused-silica capillary column. Congeners marked wih asterisks are pairs which cannot be unequivocably assigned. From Ryan *et al.* [114].

tional GC phases. The newly developed smectic liquid crystalline phase is unique in its resolving power, including the 1,2,4-substituted PCDDs. The elution order of PCDD/Fs on various smectic columns can be different, depending on the thermal history of the column. As a typical example, Fig. 6 shows GC–ECD traces of the 22 tetra-, 14 penta- and 10 hexa-CDD congeners on an SP-2331 and a liquid crystalline smectic column [114].

Naikwadi and co-workers [117,118] synthesized homopolymeric liquid crystal polysiloxane stationary phases for the separation of 2,3,7,8-substituted PCDDs, especially 2,3,7,8-TCDD. The liquid crystals have high thermal stability, low volatility (no column bleed) and are suitable for thin-layer formation in capillary columns. The separation of geometric and positional isomers gives an elution order following the length-to-breath ratio (L/B) and planarity of the solutes, so 2,3,7,8-substituted PCDDs will elute later than the other TCDDs according to the elongated structure from the lateral substitions. For furans this was not seen, presumably owing to their non-planarity. According to the authors, unique selectivity can be achieved for the separation of anthracene from phenanthrene and benzo[*a*]pyrene from benzo[*e*]pyrene.

Another column technology was found for columns coated with graphitized carbon black (GCB), a monoatomic, highly homogeneous, non-specific adsorbent whose surface is formed by basal faces of graphite. As adsorption is mainly based on the geometry and polarizability of molecules, GCB has a different elution profile from conventional liquid phases. Disadvantages of GCB are the limited me-

chanical strength of carbon particles and that, in comparison with liquid phases, higher oven temperatures need to be used owing to the high adsorption potential of carbon. Improvements were suggested by modifying the adsorption and chromatographic properties [119,120]. No applications have been published yet on the use of this type of column for the analysis of (chlorinated) aromatics in food.

3.2.2. GC–GC coupling

Multi-dimensional gas chromatography (MDGC, GC–GC) can be used as a complementary technique to resolve difficulties in the separation of co-eluting compounds in single-column GC systems. The technique is an alternative to additional sample pretreatment steps (which could lead to a further loss of analytes), and when lengthening the GC column or changing the polarity of the stationary phase (requiring time-consuming parallel injections) lead to only minor improvements in resolving power.

In GC–GC, techniques such as heart cutting (selective transfer of a group of components from the first column into the second for further separation) and backflusing (for cleaning up the first separation column from uninteresting late-eluting components by reversing the direction of flow without applying excessive temperatures) are used [123]. By using two separation systems, two sets of retention data are obtained, offering more confidence in the identification of a certain component. Initial attempts at GC–GC often suffered from bad peak shapes and a loss of sensitivity compared with conventional GC. Nowadays, the introduction of cryofocusing immediately after the transfer of cuts from the first into the second column (valve-directed switching systems) and modifications to the construction and the materials (*e.g.*, metal instead of graphite) used in the coupling device of double-oven equipped valveless systems (Sichromat 2) have led to improvements in the application of GC–GC in trace analyses of complex mixtures [124,125].

Until recently, there were hardly any practical applications in use in pesticide residue laboratories. This can be partly attributed to the fact that the technique was introduced just before the widespread introduction of capillary GC with fused-silica capillary columns. A revival of interest in GC–GC (coupling of non-chiral and chiral columns) might come as a result of new trends in the pesticide market towards the production of optically active enantiomers instead of racemic mixtures. Stronger application of GC–GC can be found in the analysis of organic micropollutants such as PCBs, PCDDs and PCDFs [125,126].

Stan and Cristall [127] reported the application of two-dimensional capillary GC with effluent splitting to three selective detectors (ECD, NPD and FPD) in the separation of γ-HCH, propachlor, propyzamide and chloro- and bromopropylate from interfering matrix compounds in samples of onions and other foodstuffs. They used a double-oven GC–GC system with a 50-m SE-54 column in the first and a 30-m DB-17 column in the second oven.

Duinker *et al.* [126] used a 25-m SE-54 column in the first oven and a 30-m OV-210 column in the second oven and applied the heart-cutting technique to separate the planar PCB congeners and, in addition, some of the mono-*ortho*-substituted analogues from closely eluting congeners. Sippola and Himberg [128] used a system consisting of a (standard) GC set-up, employing two columns (SIL-8 and HP-FFAP) connected by an SGE valve column-switching system. The technique was capable of separating the toxic PCB congeners from the complex matrix and decreased the need for sample purification.

3.2.3. SFE–GC coupling

As mentioned in Section 2.4, only a few real applications of SFE have been published and many papers demonstrate the potential of coupled SFE techniques for fatty food matrices by qualitative experiments [86,129].

Murphy *et al.* [91] reported an on-line SFE–GC–flame ionization detection (FID) determination of spiked aldrin in soybean oil. A commercial system was used, in which the chromatographic column was interfaced through a stainless-steel tee located in the oven. The restrictor, connected to the extraction cell, deposited the solutes from the extraction cell in the decompression interface region in the tee. After extraction, carrier gas was allowed to sweep the solutes from the interface to the column. By varying the extraction conditions, broader peak shapes occurred using longer extraction times and the separation of aldrin from the interfering matrix peak became worse.

A special type of coupled SFE was used by Murugaverl and Voorhees [85], who called it SFSPE–SFC, an on-line supercritical fluid extraction in combination with clean-up using solid-phase extraction followed by SFC. The SFSPE–SFC method differs from SFE–SFC in that the solid-phase trap is used for both trapping and clean-up; collection and clean-up are accomplished in one step prior to introduction of the analyte into the analytical column. Spiked fats mixed with about three parts of C_{18} sorbents were placed in the extraction cell or on top of the clean-up column and were selectively extracted, retaining the sample matrices while eluting and depositing the analytes of interest in the cryogenic trap, which could be flushed to deposit the fraction in the SFC system. Column packings were evaluated which could retain lipids and allow analytes to pass through in CO_2, which seems possible for many commercially available materials (silica, C_{18}, CN, NH_2, C_2). In spiked samples of diuron, alachlor, carbaryl in soybean oil and bendiocarb in lard, lipids were completely separated from pesticides in the first fraction (extraction for 20 min). Using a larger amount of sample (4–8 mg), some interference is shown by endogenous compounds (fatty acids) co-extracted with the pesticides. Ramsey *et al.* [86] used a similar kind of procedure, based on the differences in polarity of the extracted endogeneous material and the analytes, which could only be used for more polar analytes.

4. SPECTROSCOPIC TECHNIQUES

Through the years, spectroscopic techniques have been introduced in many laboratories for residue analyses of organic micropollutants in foodstuffs. The reason of the use of these techniques is either to obtain more selectivity and sensitivity in the trace analysis of complex mixtures or to confirm the identity of analytes following dedicated analytical techniques, usually GC or HPLC. Until recently, the well established technique of GC–MS was used almost exclusively in this field. This technique combines high sensitivity and selectivity with diagnostic structural information. However, its application range is limited to gas chromatographable compounds. Other, more recent techniques are GC coupled to Fourier transform infrared spectroscopy (GC–FT-IR) and LC–MS.

4.1. Liquid chromatography–mass spectrometry

LC-MS was first introduced in the early 1970s with the development of the moving belt interface (MBI) [137–139]. Other LC–MS techniques have been developed in the 1980s in which problems associated with the handling of relatively large solvent streams from the LC column into the mass spectrometer source have been solved in different ways. Among these, the direct liquid introduction (DLI) interface [140,141], the particle beam interface (PBI) [142] and more recently the thermospray (TSP) interface [143] have found widespread use in modern analytical chemistry. Application ranges of these interfaces differ widely from low-polarity compounds by, *e.g.*, MBI, DLI and PBI to polar compounds by TSP and electrospray. Owing to the lipophilic properties of micropollutants in biological samples and foodstuffs, LC–MS is not frequently used in this field, as LC is not often the principal method of analysis. An exception is made for PAHs and their metabolites, which are routinely analysed in some laboratories by HPLC [144,145].

The particle beam interface (equivalent terms: MAGIC, LINK) is probably the most appropriate technique for HPLC–MS of lipophilic compounds. In this interface, the LC effluent is converted into a monodisperse aerosol, the solvent is subsequently removed by a momentum separator and the heavier analyte particles are transported to the MS source where they can be ionized by electron impact (EI) or chemical ionization (CI) for structure analysis or quantitative determination. The technique is compatible with normal-bore HPLC flow-rates and good sensitivities can be achieved for low- to medium-polarity compounds.

4.2. Gas chromatography–Fourier transform infrared spectroscopy

GC–FT-IR is a relatively new technique which is recognized as very suitable for confirmation analysis of complex mixtures. Present commercially available interfaces are the light-pipe interface [146], the cryotrapping technique [147] and the matrix isolation technique in solid argon [148]. The last two techniques allow on-line analysis and spectrum averaging on the stored chromatogram trace for increased sensitivity or spectrum quality. The sensi-

tivities of both techniques are comparable down to the sub-nanogram level on-column in the full spectra mode [149]. The utility of GC–FT-IR in micropollutant analysis lies predominantly in its complementary structural information to mass spectral data for the unambiguous identification of unknowns, particularly for positional isomers. Schneider *et al.* [150] and others [151,152] have used GC–FT-IR for the analysis of chlorinated pesticides, dioxins, PCBs and PAHs in a variety of foodstuffs. Mossoba *et al.* [153] and Powell and Compton [154] used GC–FT-IR for the analysis of trace components in alcoholic beverages and foods.

4.3. Gas chromatography–mass spectrometry

As noted, GC–MS is the preferred technique where possible because of its unsurpassed separation efficiency (GC) combined with high sensitivity and specificity (MS). In regulatory practice, GC–MS is often the principal quantification method, but is frequently also used for qualitative confirmatory analysis. An example of the former is the analysis of PCDDs and PCDFs. At present, GC–MS is the only technique able to provide the required sensitivity and selectivity for trace level analyses of PCDD/Fs in biological samples. The retention parameters from GC provide isomer specificity, whereas the MS parameters provide class and homologue specificity. Clement and Tosine [155] have recently published a comprehensive review on the GC–MS analysis of PCDD/Fs. Methods include the use of non-polar and polar fused-silica capillary columns combined with low-resolution (LR) or high-resolution (HR) MS and tandem MS–MS techniques [156,157] for improved selectivity. As with dioxins, GC–MS is becoming the method of choice for trace-level analyses of PCBs in biological samples, particularly for planar and mono-*ortho*-substituted PCBs [84]. Analytical methods for PCBs include sample isotope dilution, extraction, clean-up and GC with low- or high-resolution MS. The analytical procedures for PCBs are almost identical with those used for PCDD/Fs [81]. The detection limits for PCDD/Fs and PCBs vary with the sample matrix and sample size, down to the sub-ppt level on a fat basis in biological samples and foodstuffs [69].

In contrast to quantitative analysis, confirmatory analysis is preferably performed in the full-scan mode for identification. A new, interesting technique for confirmatory analysis is ion trap detection (ITD) MS. ITD instruments contain a three-dimensional quadrupole ion storage trap first developed by Paul and Steinwedel [158] and further optimized and commercialized by Finnigan MAT [159]. In the ITD source, ions are generated and stored during the entire sample ionization time and are subsequently mass analysed. This technique provides unique sensitivity in the scanning mode, providing good-quality spectra in the low picogram range in both EI and positive-ion CI modes. A benefit of the ITD instrument is its relatively low cost. A disadvantage, however, is that the sensitivity may vary considerably when real samples are analysed owing to overloading of the trap by, *e.g.*, a high GC baseline or the presence of co-eluting interferences. This will result in a shortening of the ionization/accumulation time and hence in a lower sensitivity.

Identification and confirmation with conventional MS in the full-scan mode usually require much larger samples than are needed for single (selected) or multiple ion recording analysis (SIR). Sensitivities in the scanning mode are typically one to two orders of magnitude lower than in SIR. Therefore, SIR is frequently used for confirmatory purposes by monitoring a few ions from the analyte spectrum. However, to obtain acceptable results, protocols must have appropriate criteria for the number of ions that must be monitored, intensity ratios, the retention time and the use of different ionization techniques [160].

As noted, modern GC–MS instruments offer the opportunity of positive- and negative-ion chemical ionization (PCI, NCI) for improved molecular mass information and/or increased sensitivity. Methane and ammonia are most commonly employed as reagent gases. PCI has been frequently used for pesticide analysis. Many of these compounds undergo strong fragmentation under EI with low-abundant molecular ions. Methane or ammonia CI often generate quasi-molecular ions, $[MH]^+$. Cairns *et al.* [161] have constructed a molecular mass list of the majority of pesticides as an aid for the identification of suspected pesticide residues.

In the analysis of PAHs, CI will generally not provide more additional information than EI, because EI spectra usually contain abundant molec-

ular ions. Difficulties with the identification of PAHs lies predominantly in the lack of diagnostic differences in the EI spectra of isomers. Additional information of GC retention parameters is often needed for identification. The selectivity for isomeric PAHs can be altered by the use of other ionization techniques. Hilpert [162] showed that the sensitivities of different PAHs to negative-ion CI differ greatly, which provided an easy method to discriminate between isomeric PAHs and alkylated PAHs. For example, fluoranthenes were sensitive in NCI, whereas pyrenes were almost transparent. Sim *et al.* [163] compared LC (moving belt) and GC, both combined with MS, for the determination of PAHs. Further complementary separation methods were not needed for complete separation and identification of a complex mixture. The higher column selectivity in HPLC was used for the determination of isomeric compounds. Differentiation between isomers was studied by Brotherton and Gulick [164] using hydrogen PCI. Others used charge transfer in the presence of argon–methane mixtures in the source [165].

NCI, particularly electron-capture negative-ion CI [166], has been found to be useful for increased sensitivity for the analysis of many micropollutants. The sensitivity of compounds under NCI often parallels that of the ECD in GC. In the NCI process, near-thermal electrons are generated in the source under high-pressure CI conditions (0.2–1 Torr), and rapidly react in relatively high yields with suitable molecules to produce M^- ions. Further fragmentation may occur, depending on the structure and the experimental and instrumental conditions [167]. The highest sensitivity is usually achieved for compounds with conjugated or aromatic structures with a sufficient number of halo substituents. Hexachlorobenzene (HCB), for example, is extremely sensitive in NCI. Stan and Kellner [168] examined 72 organophosphorus pesticides under PCI and NCI conditions and found 59 to be more sensitive in NCI and 13 in PCI.

Other polychlorinated micropollutants such as PCBs and PCDD/Fs can be analysed using NCI-MS. Characteristics of the NCI mass spectra of PCDD/Fs have been extensively studied [169]. The fragmentation and sensitivity depend on the degree of chlorination and the substitution pattern. For instance, 2,3,7,8-TCDD was found to be less sensitive than the other TCDDs [170] and PCDFs were more sensitive than PCDDs. Significant differences in spectra were observed depending on the operating conditions, the most critical being the source temperature [167] and the presence of oxygen in the ion source [171]. Traces of oxygen lead to the formation of O^- and O_2^- ions, which can react with molecular ions to give $[M\text{-}19]^-$ by displacement of Cl by O [172].

In summary, research over the last few decades has resulted in the development of impressive methodologies for the characterization, identification and highly sensitive determination of micropollutants in foodstuffs by spectroscopic techniques. Trace-level organochlorine pesticides can be detected and identified by capillary GC combined with EI-, PCI- and NCI-MS. Current methods for PCDD/Fs and PCB analysis are well established and are highly sensitive and specific at the sub-ppt level. Their performance is unmatched by other analytical techniques. Future research to improve GC–MS analyses of brominated or mixed bromo–chloro PCDD/Fs will primarily focus on the isomer-specific separation of the much larger number of toxic congeners. LC–MS and GC–MS methods using different ionization techniques are available for the identification of PAHs in complex samples, but discrimination remains difficult. A significant contribution to the solution of this problem is foreseen by the use of GC–FT-IR, which will find increased application in the field of micropollutant analysis, particularly for the identification of isomers.

5. CONCLUSIONS

Although the determination of apolar organic micropollutants in lipid materials is a well established field with its first publications originating from the 1950s, much research is still needed for the development and improvement of residue analysis. The methodology described in this paper is used by governmental laboratories for risk assessment and regulatory practice. For this reason, method development should mainly be focused on improvements of the sample throughput and reliability. High plate numbers in a novel miniaturized chromatographic column may seem to be very attractive from a theoretical point of view, but the final outcome that

counts is whether the system will be able to produce a large amount of reliable data in a minimum amount of time.

Over the years, attention has shifted from the OCPs to PAHs and PCBs, ultimately resulting in major efforts in the late 1980s to develop sensitive methods of analysis for PCDDs and PCDFs. At the same time chromatography has developed strongly, and a major trend towards coupled techniques can be observed. Coupling of chromatographic techniques with other chromatographic techniques (LC–GC, SFE–GC, GC–GC, LC–LC) greatly alleviates the problem of manual sample pretreatment and enhances selectivity and sensitivity. Coupling of chromatographic techniques with spectroscopic techniques (GC– and LC–MS, GC– and LC–FT-IR) gives a new dimension to the original chromatographic techniques. The use of GC–MS has greatly facilitated the development of ultra-trace-level methods for PCDDs, PCDFs and for the toxicologically related planar PCBs.

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Review

Determination of aflatoxins in food products by chromatography

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ABSTRACT

Several chromatographic methods for the determination of aflatoxins in agricultural and food products are reviewed. During the past two decades, identification and determination of aflatoxins were done by thin-layer chromatography (TLC) because it was easy, fast and inexpensive. However, high-performance liquid chromatography (HPLC) using fluorescence detection is now the method of choice for determining aflatoxins and is also growing in popularity for their identification. The reasons for selecting HPLC over TLC can be summarized as the ability to analyze for a wide variety of compounds, including compounds that are easily degraded by heat, light or air, the ease of adaptation to confirmatory procedures, the potential for automation and the dramatic improvement in instrumentation, including the development of increasingly sensitive fluorescence and electrochemical detectors and short, high-resolution, reversed-phase columns.

CONTENTS

1. INTRODUCTION

Ever since the severe outbreak of Turkey "X" disease in the UK in the 1960s and the discovery of fluorescent compounds in a feed component, peanut meal, aflatoxins have been a major concern as a toxic contaminant of feeds and foods [1]. Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The word "aflatoxin" was derived from "a" from the genus *Aspergillus*, the "fla" from the species *flavus* and "toxin" meaning toxic [2]. Cole and Cox [3] listed sixteen compounds in the aflatoxin group, but only the aflatoxins B_1, B_2, G_1, G_2 and M_1 are routinely monitored in foods and feeds in commerce (Fig. 1). The International Agency for Research on Cancer has placed aflatoxin B_1 on their list of probable human carcinogens [4]. The fungi can produce aflatoxins on commodities in the field under stress conditions or in storage when conditions such as high moisture and warm temperature (25–30°C) are met [5].

Aflatoxin B_1 is the prevalent, acutely toxic and most carcinogenic of the aflatoxins and M_1 is excreted in the milk of animals ingesting aflatoxin B_1 [5]. Because of potential health hazards for humans, worldwide monitoring of aflatoxins in various commodities has been indicated an regulatory levels in different commodities have recently been documented [6]. Efforts to minimize aflatoxin contamination in susceptible commodities are the subject of research projects in many places.

Aflatoxin determination is no longer a particularly difficult task using current thin-layer chromatographic (TLC), high-performance liquid chromatographic (HPLC) and immunochemical techniques. Aflatoxins B_1, B_2, G_1, G_2 and M_1 can be readily separated and detected using either normal- or reversed-phase TLC or HPLC techniques, with HPLC becoming increasingly the method of choice.

The challenges inherent in aflatoxin analysis currently include sampling, subsampling and sample extraction methods and also the analytical variation associated with the chosen analytical method.

For this review, selected examples of techniques have been used, and therefore the literature cited is not exhaustive. For more information on specific techniques, the reviews by Rottinghaus [7], Beaver and Wilson [8], Wilson [9], Wilson *et al.* [10], Betina [11] and Beaver and Wilson [12] should be consulted.

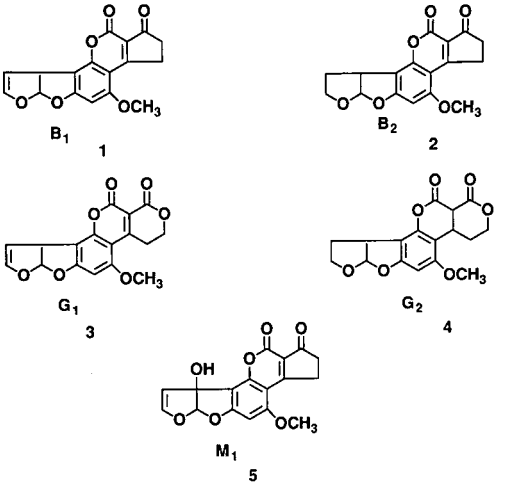

Fig. 1. Structures of the major aflatoxins. 1 = aflatoxin B_1; 2 = aflatoxin B_2; 3 = aflatoxin G_1; 4 = aflatoxin G_2; 5 = aflatoxin M_1.

2. SOLVENT EXTRACTION

Extraction of aflatoxins from various sample matrices has been accomplished using several organic solvents. Aqueous mixtures of methanol, acetonitrile, 2-propanol or acetone have been used for extraction of grains, oilseeds, cottonseed, nuts, meats and various other products. In most instances a two-phase extraction is carried out using chloroform–water or methanol–water.

Three different solvents were developed for aflatoxin extraction from peanut, cottonseed and corn. The AOAC CB method [13] uses chloroform saturated with water as the extraction solvent and is the standard by which other methods are judged. The CB method works very well with corn and peanuts but many interfering substances are seen when this method is used for cottonseed or mixed feeds. The other disadvantages of chloroform extraction include the expense of the solvent, toxicity and problems with waste disposal. The AOAC BF method [14], developed for TLC analysis of peanuts and peanut products, uses a methanol–water (55:45) extraction with hexane added to defat the sample. Aflatoxins are partitioned into chloroform prior to the TLC determination. Pons developed the AOAC cottonseed products method [15] which uses an acetone–water (85:15) extraction followed by a lead acetate precipitation, filtration, partitioning into dichloromethane, clean-up on a silica gel column and determination by TLC or HPLC. Acetonitrile plus water in various ratios has also been used frequently as the extraction solvent for aflatoxin M_1 and for multi-mycotoxin methods.

Shotwell and Goulden [16] compared the extraction efficiencies of the BF method and the AOAC cottonseed method with that of the CB method in corn. The BF method uses methanol–water (55:45) and the cottonseed method uses acetone–water (85:15) as extraction solvents. Neither of these solvents extracted aflatoxins from corn as efficiently as the CB chloroform–water (250:15) extraction. Bradburn *et al.* [17] evaluated aflatoxin extraction from corn using differing concentrations of aqueous acetone, aqueous methanol and aqueous acetone–methanol (1:1) as extraction solvents. With each system the amount of aflatoxin extracted increased as the ratio of organic solvent to water increased from 50:50 to 80:20 and then decreased or remained constant at 90:10. Aqueous acetone (80%) was found to extract 27% more aflatoxin than the corresponding 80% methanol, with 80% methanol–acetone (1:1) being intermediate. Hurst *et al.* [18] used methanol–water (55:45) as an extraction solvent for aflatoxins in peanut butter. Leitao *et al.* [19] used chloroform–water (10:1) in the determination of aflatoxins in various strains as *Aspergillus* in foodstuffs. Groopman and Donahue [20] used methanol–water (60:40) for extraction prior to affinity column clean-up for the determination of aflatoxins in foods and biological samples. The AOAC accepted method of extraction using methanol–water (60:40) was utilized by Dorner and Cole [21] in the analysis of peanuts after mini-column clean-up and by Boyacioglu and Gonul [22] in the analysis of raisins.

Selection of a particular extraction solvent depends on the type of chromatographic detection. Many other factors, including commodity, stability, interfering substances, cost and waste disposal, must be taken into consideration before selecting a method. In general, chloroform or dichloromethane is acceptable with the exception of immunochemical method which are not sensitive to chlorinated hydrocarbons. The immunochemical methods generally use various methanol–water extraction solvents. Matrix and sensitivity considerations should be the most important factors influencing the selection of extraction solvents.

3. SCREENING METHODS

In many instances only qualitative identification of the aflatoxins present in various media is needed. Ideally, the screening method should allow a determination of whether aflatoxins are present above a specific level without any sample preparation. The food and feedstuffs most frequently contaminated with aflatoxins are peanuts, cottonseed, corn and tree nuts. The simplest aflatoxin screening method is the use of black or long-wavelength UV radiation (365 nm) to examine cracked or coarsely ground corn. However, the USDA is considering halting the use of this method because of difficulty in interpreting the observed bright greenish yellow fluorescence (BGYF).

More reliable screening methods such as enzyme-linked immunosorbent assay (ELISA) and TLC normally involve and initial solvent extraction step prior to analysis.

3.1. Enzyme-linked immunosorbent assay (ELISA)

The development of commercially available ELISA kits that recognize different mycotoxins has made immunoassay an important tool for aflatoxin testing. The typical ELISA format for aflatoxins contains three specific reagents: the mono- or polyclonal antibodies that recognize a specific mycotoxin such as the aflatoxins and bind with them, an aflatoxin–enzyme conjugate and an enzyme substrate. Binding of the aflatoxin–enzyme conjugate by immobilized antibodies is prohibited by the presence of free toxin in the sample. The bound enzyme catalyzes the oxidation of a substrate to form a colored complex. Development of color indicates that the test sample contains no aflatoxin or a concentration below the level of interest. Two ELISA methods for aflatoxins were collaboratively studied. One method used microtiter wells [23] and the other used a membrane attached to a plastic cup [24]. The microtiter well method had minor problems and is being modified. The microtiter well method can also be used for semi-quantitative determinations when an ELISA reader is utilized to record the absorbance of the colored complex.

3.2. Immuno-dot

The cup or immuno-dot method is a "yes–no" test. Performance is assessed by examining the ability of the test to classify samples correctly into two categories, positive and negative at a predetermined detection level (20 ng/g) for the aflatoxins. Ideally, above 20 ng/g all results should be positive and all below should be negative. The 95% confidence intervals for correct identification of cottonseed and peanut butter as positive for aflatoxin contamination at 20 ng/g using this method were 79–100%, and for raw peanuts and corn 78–100% and 73–99%, respectively [24].

3.3. Thin-layer chromatography (TLC)

The most effective screening method uses TLC, which is the simplest of all the widely used chromatographic methods to perform. A developing tank containing the mobile phase, a coated plate and long-wavelength UV radiation are all that are required for separations and qualitative analyses. TLC of the aflatoxins has received the most attention over the years; consequently, it is the most refined and generally serves as a model for other mycotoxins. Most analysts prefer commercially prepared silica gel plates because of durability and homogeneity of the adsorbent layer. After sample application the plate is put in a developing tank containing mobile phase. The most commonly used mobile phases for aflatoxin analyses are chloroform–acetone (9:1), diethyl ether–methanol–water (96:3:1) and anhydrous diethyl ether.

A portion of a sample extract is evaporated and the oily residue along with a standard solution of aflatoxins are spotted on a TLC plate and developed with anhydrous diethyl ether [25]. The lipids move to the solvent front, while the four aflatoxins are separated. The plate is then examined under long-wavelength UV light. This method can easily detect aflatoxins at a level of 20 ng/g. The rate of correctly detecting contamination is 80% at 20 ng/g.

4. SAMPLE CLEAN-UP

There are three steps in the analytical procedures: extraction, purification and determination or analysis. The most common solvent system for extraction is a mixture of a chlorohydrocarbon and water, but is gradually being replaced with methanol–water or acetonitrile–water systems. The most significant improvement in the purification step is the use of solid-phase extraction (SPE). Clean-up of sample extract prior to instrumental analysis (TLC or HPLC) is used to remove other materials also extracted that often interfere in the determination of target analytes. The traditional use of column chromatography (silica gel) and liquid–liquid partition for clean-up has been relaced by SPE. SPE is rapid, solvent efficient and economical. The most commonly used stationary phases in SPE columns are silica gel [26], C_{18} bonded-phase [27], Florisil [28], Mycosep multi-functional clean-up [29] and antibody affinity types [30].

4.1. Silica gel columns

Wei *et al.* [31] used Sep-Pak silica cartridges in conjunction wih preparative TLC using silica gel to clean-up CB extracts of soy sauce and fermented soybean paste for HPLC determination of aflatoxins. Cohen and Lapointe [32] determined aflatoxins in corn and dairy feed using HPLC and fluorescence detection after clean-up with Sep-Pak silica

cartridges. The corn and dairy feed samples were extracted initially with acetonitrile–water and the aflatoxins were partitioned in chloroform prior to Sep-Pak silica SPE.

Trucksess *et al.* [33] used a 0.5-g disposable silica gel column to clean-up methanol–water extracts of corn and peanut butter after the extract had been partitioned with chloroform. Aflatoxin analyses were performed by TLC with gas chromatographic–mass spectrometric (GC–MS) confirmation. Hurst *et al.* [34] analyzed raw peanuts for aflatoxins by HPLC after the acetone–water extracts had been partitioned in dichloromethane and cleaned up by passage through Sep-Pak silica cartridges.

Tyczkowska *et al.* [35] used liquid chromatography to analyze for aflatoxin M_1 in milk after clean-up with Sep-Pak silica cartridges. The milk was extracted with acetone, defatted with hexane and the aflatoxin M_1 was partitioned in chloroform prior to Sep-Pak silica clean-up.

Hutchins *et al.* [36] evaluated a rapid Sep-Pak silica clean-up for the determination of aflatoxins in corn using HPLC and fluorescence detection after precolumn derivatization with trifluoroacetic acid. The corn samples were extracted with chloroform–water and mixed with hexane prior to clean-up on Sep-Pak silica cartridges. The results indicated that recoveries for the aflatoxins were greater than 97% overall. The working range for this method was from <1 ng/g to >100 μg/g of aflatoxin B_1.

It should be mentioned, however, that utilizing silica gel for clean-up involves the use of potentially hazardous solvents such as chloroform.

4.2. Florisil columns

Kamimura *et al.* [37] purified chloroform–water extracts from corn, buckwheat, peanuts and cheese with a Fluorisil column and determined the aflatoxins utilizing high-performance TLC (HPTLC). Van Egmond *et al.* [27] compared six different methods for determining aflatoxin B_1 in feeding stuffs containing citrus pulp, and the preferred method involved purifying the chloroform extracts with Sep-Pak Florisil cartridges and Sep-Pak C_{18} cartridges before HPLC analyses. This method was recommended to the European Community (EC) for adoption following the results of a collaborative study in 1991 [38].

4.3. Mycosep multi-functional clean-up columns

Multi-functional clean-up (MFC) columns provide a rapid one-step extract purification. These columns work in just the opposite way to other clean-up columns. The MFC columns are designed to allow compounds of interest to pass through, while retaining compounds that could create interferences in most analytical methods. Wilson and Romer [29] purified acetonitrile–water extracts from several agricultural food products using MFC columns prior to HPLC analysis. Aflatoxin recoveries reported were above 95% with a sensitivity of <1 ng/g.

Romer [39] is planning to market a method for determining aflatoxins that is completely automated. The method involves on-line clean-up of sample extracts with MFC columns and HPLC analysis using fluorescence detection after postcolumn derivatization with bromine.

4.4. Monoclonal antibody affinity columns

Groopman and Donahue [20] used monoclonal antibody affinity columns to isolate aflatoxins rapidly from food and grain samples and aflatoxin M_1 from milk. Portions of methanol–water extracts were diluted and passed through the affinity columns. The aflatoxins were eluted from the column with methanol and subquently analyzed using reversed-phase LC. Holcomb and Thompson [40] used affinity columns to isolate aflatoxins from rodent feed prior to analysis by HPLC using fluorescence detection after postcolumn derivatization with iodine.

Farjam *et al.* [41] used an immuno precolumn packed with monoclonal or polyclonal antibodies for on-line analysis of aflatoxin M_1 in defatted milk. Mortimer *et al.* [42] used immunoaffinity column clean-up for aflatoxin M_1 in milk and analysis by HPLC.

As affinity columns isolate the aflatoxins from virtually all interfering compounds, sensitivities of <1 ng/g can easily be obtained.

4.5. Gel permeation chromatography

The use of gel permeation chromatography (GPC) for clean-up purposes has not been extensively utilized. However, GPC has the potential and has been used to purify sample extracts for aflatoxin analysis. Hetmanski and Scudamore [43] used GPC with a column packed with Bio-Beads S-X3 to clean

up extracts of cereals and animal feedstuffs prior to analysis by HPLC. Quantitative results were obtained down to 1 ng/g.

5. THIN-LAYER CHROMATOGRAPHIC QUANTIFICATION

Quantitative tests, with high precision and accuracy, have become a reality because of the improvements in instrumentation and the availability of a wide variety of adsorbents for use as stationary phases on TLC plates. Stationary phases have made great advances in recent years. Media of small particle size with a narrow size distribution have become available. HPTLC plates are made of such media. Various instrumentation for sample application, plate development and densitometry has recently been evaluated [44]. Optimum sensitivity, accuracy and precision were obtained by using a fully automated TLC sampler, an unsaturated conventional TLC glass chamber and a monochromatic fluorodensitometer. Recently a microcomputer was interfaced to a fluorodensitometer to simplify the data-handling procedure [45]. The system computes and records the amount of aflatoxin in the sample extract spots and the concentration of aflatoxin in the original extracted sample. TLC has maintained its analytical status because of the constant improvements in instrumentation and stationary phases.

5.1. One-dimensional

One-dimensional TLC includes one solvent, two solvents and bidirectional development systems. The one-solvent system is self-explanatory. In two-solvent development [46], first the plate is developed with a solvent that removes the interferences and then the plate is dried and developed with another solvent in the same direction for the separation of the toxins. In bidirectional development [47], sample extracts are spotted in the middle of the plate. After the first development with a non-polar solvent to remove the non-polar compounds, the top of the plate below the solvent front is cut off. The plate is then turned upside down (180°C) and developed with a more polar solvent to separate the toxins.

5.2. Two-dimensional

Two-dimensional TLC (2D-TLC) is the most powerful technique and offers greater resolution than other chromatographic techniques. It uses two solvents of different selectivity for the two developments. The usefulness of 2D-TLC for aflatoxin analysis has been demonstrated in a number of recent publications [26,27,48–50].

6. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTIFICATION

HPLC using fluorescence detection has already become the most accepted method for the determination of aflatoxins. HPLC is also fast becoming the method of choice for qualitative analyses of aflatoxins because of their native fluorescence of λ_{ex} = 360 nm for all four aflatoxins and λ_{em} = 440 nm for aflatoxins B_1 and B_2 and 470 nm for aflatoxins G_1 and G_2 [51].

HPLC methodology has several advantages over other methods but the most important seems to be the potential for automation. For a thorough review of HPLC data for aflatoxins, the surrey by Shephard [52] should be consulted.

Both normal- and reversed-phase HPLC can be utilized. Normal-phase methods have been developed using detection by UV monitoring at 254 and 365 nm, native fluorescence and fluorescence with a silica packed cell. Reversed-phase methods using both UV and fluorescence detection have been developed. Recently, reversed-phase HPLC methods for aflatoxins have received the most attention. A review of these methods is given.

6.1. Normal-phase

The early work of Pons [53] with applications chemists at DuPont resulted in the development of a normal-phase separation using a water-saturated chloroform–cyclohexane–acetonitrile–ethanol mobile phase. The initial separation was accomplished and aflatoxins B_1, B_2, G_1 and G_2 could be detected at either 254 or 365 nm using a UV detector. Kmieciak [54] reported that UV detection between 350 and 360 nm was more selective than at 254 nm. Garner [55] found that a silica gel column packed with material of 6-μm mean particle size was more efficient in separating aflatoxins than a column packed with particles of size ranging from 5 to 10 μm.

Pons and Franz [56,57] developed methods for

cottonseed and peanut products using normal-phase chromatography with UV detection at 365 nm. Pons and Franz [57] also determined that the use of UV detection at 365 nm was to be preferred for detection of aflatoxins B_1 and B_2 whereas fluorescence detection (excitation at 365 nm and emission above 450 nm) was preferred for aflatoxins G_1 and G_2.

The use of fluorescence detections has been hampered because the emission of the fluorescence of aflatoxins B_1 and B_2 is quenched by mobile phases containing chlorinated solvents. Two alternative methods were developed to overcome this limitation. First, the use of alternate mobile phases was investigated and second, a silica gel-packed flow cell was developed to enhance the fluorescence with normal-phase solvents.

Chang-Yen *et al.* [58] reported that the fluorescence of aflatoxins B_1, B_2, G_1 and G_2 was influenced by the solvent composition with various chloroform–methanol combinations. This dependence on solvent composition has limited the use of chloroform-based mobile phases without some type of fluorescence enhancement.

Manabe *et al.* [59] reported that a mobile phase consisting of toluene–ethyl acetate–formic acid–methanol (89:7.5:2.0:1.5) did not quench the fluorescence of aflatoxins B_1 and B_2 whereas mobile phases with chloroform, dichloromethane or methanol as the major components did quench the fluorescence. They reported that the relationship between peak area and concentration was linear up to 120 ng and that the method was sensitive to 10–20 ppb of total aflatoxins. Goto *et al.* [60] used toluene–ethyl acetate–formic acid–methanol (90:5.0:2.5:2.5) as the mobile phase to separate aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 and developed a method with high recovery for the analysis of milk and milk products. Leitao *et al.* [19] also used a toluene–ethyl acetate–formic acid–methanol (90:6:2:2) mobile phase for aflatoxin determination in *A. flavus* group culture extracts. Howell and Taylor [61] used water-saturated toluene–ethyl acetate–formic acid (85:25:5) as the mobile phase to determine aflatoxins in mixed feeds using fluorescence detection with a limit of 1 ng/g. They changed the excitation wavelength from 360 to 330 nm with a consistent emission at 425 nm for confirmation. The 365/330 nm peak-height ratio from positive samples agreed within 10% of standard values for aflatoxins B_1, B_2, G_1 and G_2 [61]. Perhaps other solvent systems could be identified that improve the detectability of aflatoxins in normal-phase HPLC without adding a step to enhance the fluorescence.

The primary fluorescence enhancement technique for normal-phase HPLC has been to use a detector flow cell packed with silica gel. The silica gel-packed flow cell was developed by Panalaks and Scott [62] and was reported to be linear up to about 2 ng injected. A normal-phase HPLC method was developed by Pons [63] for corn using a methanol–10% aqueous sodium chloride extraction, precipitation with zinc acetate and preliminary clean-up using a small silica gel column and detection using a packed flow-cell. Pons [63] compared the methanol–10% aqueous sodium chloride extraction with the CB extraction in samples containing only aflatoxins B_1 and B_2 and reported that the results were essentially identical, while methanol–10% aqueous sodium chloride extracted more aflatoxins when the samples contained aflatoxins B_1, B_2, G_1 and G_2. The average recovery was above 90% for all aflatoxins tested. Awe and Shranz [64] and Francis *et al.* [65] developed methods for spices and peanut butter using a silica gel packed flow cell. The AOAC method [15] for cottonseed products uses normal-phase HPLC with detection either with UV monitoring at 365 nm or fluorescence detection with a packed cell.

6.2. Reversed-phase

Development of reversed-phase HPLC methods for aflatoxin determination was similar to normal-phase HPLC development. The use of reversed-phase methods is now more common than that of normal-phase methods. Seitz [66] investigated the use of dry packed octadecyl and phenyl reversed-phase columns and found that they were incapable of resolving the aflatoxins. Hurst and Toomey [67] used a 5-μm reversed-phase C_{18} column to develop a method for the determination of aflatoxins in peanut products. The eluate from the column was passed to a UV detector operated at 365 nm to determine the aflatoxins and then into a fluorescence detector to improve the accuracy aflatoxin of B_2 and G_2 determination. Hurst *et al.* [68] also developed a reversed-phase method for cocoa beans using UV detection at 365 nm for aflatoxins B_1 and G_1 and fluorescence detection of aflatoxins B_2 and

G_2. A silica gel-packed flow cell was used by Knutti *et al.* [69] for the determination of aflatoxins in peanut kernels. The packed cell enhanced the fluorescence signals of aflatoxins B_1 and G_1, eliminating the need for dual detectors.

A high-affinity monoclonal antibody specific for aflatoxins was used in an immunoaffinity column by Groopman *et al.* [70] and Groopman and Donahue [20] in methods developed for determination of aflatoxin metabolites in urine and aflatoxins in foods. They used a 5-μm ODS reversed-phase column and a UV detector with isocratic elution with 18% ethanol for 20 min followed by an 18–25% ethanol gradient over 25 min. The mobile phase was buffered at pH 3.0. The common aflatoxins and several aflatoxin metabolites were separated and determined using this technque.

UV detection has the disadvantage of not being as sensitive or selective as fluorescence detection and interfering peaks are more common. Therefore, methods development has focused on the more sensitive and selective fluorescence detection with excitation at about 365 nm and emission at about 440 nm.

A kinetic study of the acid-catalyzed conversion of aflatoxins B_1 and G_1 and B_{2A} and G_{2A} was published by Pons *et al.* [71] in 1972. The rate of conversion of aflatoxins B_1 and G_1 to the corresponding saturated hydroxy derivatives, B_{2A} and G_{2A}, was found to be first order and strongly pH dependent. This work formed the basis for the derivatization of aflatoxins B_1 and G_1 to B_{2A} and G_{2A} with trifluoroacetic acid on TLC plates or in test-tubes prior to HPLC separation. In reversed-phase HPLC mobile phases aflatoxins B_1 and G_1 are not highly fluorescent whereas B_{2A} and G_{2A} are easily detected. The conversion of aflatoxins B_1 and G_1 to B_{2A} and G_{2A} was accomplished by Takahashi [72,73].

Takahashi [72] developed an HPLC method for the determination of aflatoxins in wines and other liquid, using a 10-μm ODS column with water–acetonitrile–methanol (15:3:2) as the mobile phase. Aflatoxins B_1 and G_1 were converted into B_{2A} and G_{2A} before injection, whereas B_2 and G_2 were not affected by the treatment with trifluoroacetic acid. The recovery of aflatoxins B_1, B_2, G_1 and G_2 from various liquid products spiked at 1 ng/ml was 80–116% with the detection limit being about 0.02 ng/ml. Reversed-phase methods using precolumn derivatization were soon developed for green coffee and peanut butter [74], corn and dairy feeds [32], pistachio nuts [75] and animal feeds [50]. De Vries and Change [76] reported a correlation of 0.991 between reversed-phase HPLC using precolumn derivatization and the CB method with corn and peanut butter. Tarter *et al.* [77] published an improved method using precolumn derivatization and reversed-phase HPLC which was suitable for use with peanut, various tree nuts and pumpkin seed. This method was used in a successful AOAC collaborative study on corn and peanut butter. This new AOAC method [78] for corn and peanut butter uses precolumn trifluoroacetic acid derivatization with separation on an RP-C_{18} column accomplished using water–acetonitrile–methanol (700:170:170) as the mobile phase and fluorescence detection (excitation at 360 nm, emission at 440 nm).

The major problem in the chromatography of aflatoxins B_{2A} and G_{2A} is the relative instability of these compounds. Both are very unstable in methanol and precautions must be taken to protect their stability in injection solvents. Usually this is accomplished with an acetonitrile–water injection solvent. The low stability of aflatoxins B_{2A} and G_{2A} can present major problems when samples are placed in an autosampler hours before injection. Therefore, precautions must always be taken when using methods for aflatoxins B_{2A} and G_{2A}.

The use of precolum derivatization in HPLC separation also introduced an additional step in the analysis. Postcolumn enhancement of fluorescence is desirable because it offers and easily controlled in-line step. Davis and Diener [79] reported that the iodine derivative of aflatoxin B_1 was 25 times more fluorescent than the parent aflatoxin. The structures of the iodine derivatives of aflatoxins B_1 and G_1 were not determined because the derivatives appeared to be unstable. However, their observations led to the development of a postcolumn iodine derivatization method by Thorpe *et al.* [80]. Tunistra and Haasnoot [51] improved this method and Shepherd and Gilbert [81] investigated conditions for postcolumn iodination for the enhancement of aflatoxin B_1 fluorescence. Shepherd and Gilbert [81] reported that optimum postcolumn iodination with iodine saturated water solutions consisted of a 5000 × 0.3 mm I.D. reactor coil operated at 75°C with a

reagent flow-rate of 0.5 ml/min. The HPLC used a 5-μm Spherisorb ODS column maintained at 35°C and eluted with water–acetonitrile–methanol (60:30:10) at 0.75 ml/min with fluorescence detection (excitation at 365 nm, emission at 440 nm).

Theil *et al.* [82] developed a postcolumn iodine reversed-phase method for determining aflatoxins in corn, peanut butter, sorghum malt and duckling mash. The separation and conditions were similar to those developed by Shepherd and Gilbert [81]. The iodine solution was prepared daily and confirmation of aflatoxins B_1 and G_1 was accomplished by stopping the iodine flow and observing the disappearance of the B_1 and G_1 peaks. Methods using postcolumn iodination were developed by Hurst *et al.* [34] for peanut products, by Paulsch *et al.* [83] for feedstuffs containing citrus pulp and by Chamkasem *et al.* [84] for multi-mycotoxin screens in grains, oilseeds, and animal feeds. Beaver *et al.* [85] compared postcolumn iodine derivatization with the CB TLC method and found a correlation coefficient of 0.99 for aflatoxin B_1.

An AOAC–IUPAC collaborative study was conducted by Trucksess *et al.* [86] to evaluate the Aflatest immunoaffinity column for the determination of aflatoxins in corn, peanuts and peanut butter. The method used reversed-phase HPLC with postcolumn iodine derivatization. The samples were extracted with methanol–water (7:3) and diluted to $<30\%$ methanol before application to the affinity column. The column was washed with water before elution with 1 ml of methanol. The methanol solution was diluted to 2 ml with water and 50 μl were injected into the HPLC system. The chromatographic conditions were similar to those of Shepherd and Gilbert [81] except that a 5-μm C_{18} column was generally used with a mobile phase consisting of water–acetonitrile–methanol (3:1:1). Recoveries were 81, 81 and 83% for samples spiked at 10, 20 and 30 ng/g, respectively. The collaborative study results were acceptable for within-laboratory and between-laboratories precision and the method was adopted as an AOAC–IUPAC method [87]. The major disadvantages of the postcolumn iodine method are the need for daily preparation of the iodine solution and the necessity for two pumps.

The development of a method using postcolumn derivatization with electrochemically generated bromine by Kok and co-workers [88,89] overcomes the need for a second pump but adds an electrochemical KOBRA cell for the generation of bromine. Kok *et al.* [88] described the optimum operating characteristics of the KOBRA cell and applied this technique in a method for determining aflatoxins in cattle feed [89]. An RP-C_{18} column was used to accomplish the chromatographic separation with a mobile phase of water–methanol–acetonitrile (13:7:4) containing 1 m*M* potassium bromide and 1 m*M* nitric acid. The reaction coils provided reaction times of 4, 8 and 24 s at a flow-rate of 0.5 ml/min. The aflatoxins were detected using a fluorescence detector (excitation at 360 nm, emission at >420 nm). The detection limits were reported to be 0.04 ng for aflatoxins B_1 and G_1 and 0.02 ng for B_2 and G_2. Trucksess *et al.* [90] evaluated the KOBRA cell reversed-phase method using corn with three different clean-up procedures. TLC results were similar to HPLC results and the HPLC method could be automated.

7. GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC CONFIRMATION

Confirmation of the identity of aflatoxins by means of MS analysis in the past required additional clean-up such as TLC isolation or SPE [91], as the presence of impurities in the sample extracts caused problems. New approaches involve interfacing gas chromatography with mass spectrometry (GC–MS), which uses GC to separate the impurities in the extracts and MS to confirm the identities of the aflatoxins. Trucksess *et al.* [92] were the first to use GC–MS to analyze for aflatoxin B_1. The extract samples were injected directly on to the GC column at 40°C. Immediately after sample injection, the column temperature was raised to 250°C in 4 min. A 6 m × 0.2 mm I.D. methylsilicone-coated fused-silica column was used and the effluent was analyzed by negative-ion chemical ionization (NICI) MS.

Goto *et al.* [93] used GC with flame ionization detection to analyze a mixture of four aflatoxins. The initial and final temperatures were set at 50 and 300°C, respectively, and the rate of heating was set at 15 or 20°C/min. A 5% phenylmethylsilicone capillary column was used to separate aflatoxins B_1, B_2, G_1 and G_2 with 2, 2, 4 and 4 ng being injected, respectively. This technique coupled with MS could be used for the determination and confirmation of the aflatoxins.

Holcomb *et al.* [94] used thermospray mass spectrometry (TSP-MS) to characterize the reaction products of aflatoxins B_1 and G_1 with iodine in methanol–water. About 2 μg of the derivatives were injected into the HPLC–TSP-MS system. The mass spectra showed ions at *m/z* 471 and 488, corresponding to the $[M+H]^+$ ion of the derivatized aflatoxins B_1 and G_1, respectively. These results indicated that the reaction products were adducts with one iodine atom and a methoxy group on the furan ring. Hurst *et al.* [95] used an HPLC–TSP-MS method for the confirmation of aflatoxins in peanuts. A 5-μm C_{18} column (25 cm × 4.6 mm I.D.) was used with a mobile phase of 0.1 *M* ammonium acetate–methanol–acetonitrile (56:22:22) at a flow-rate of 1.0 ml/min. The detection limits for B_1, B_2, G_1 and G_2 were 60, 40, 100 and 100 pg, respectively.

8. FUTURE PROSPECTS AND CONCLUSION

Methods for the determination of aflatoxins by TLC and HPLC have been well developed. The separation and determination of aflatoxins B_1, B_2, G_1, G_2 and M_1 in various matrices are no longer difficult. The individual aflatoxins can be routinely determined in almost any well equipped laboratory. The fact that the aflatoxins are fluorescent and can be selectively analyzed has helped in methods development. The advent of immunochemical technology has made aflatoxin detection possible in many different environmental conditions.

The primary difficulty with aflatoxin analysis lies with sampling and subsampling and not with the analytical method. Whitaker *et al.* [96] calculated the sampling, subsampling and analytical variances associated with testing corn. At 20 ng/g, the relative standard deviations associated with a 4.5-kg sample a 1-kg coarse-ground subsample, a 50-g fine-ground analytical sample and one CB TLC analysis were 21, 8, 11 and 26%, respectively. The use of HPLC or other more precise methods could reduce the variation of the analytical step to below 5% [97].

The greatest need is to improve the methods for sampling, subsampling and analytical sample preparation in order to reduce overall variations and make determinations of aflatoxin content in a given lot more accurate. Only limited improvements may be possible because of the heterogeneous distribution of aflatoxins in contaminated food and feed. At present, these sources of error far outweigh the variance contributed by the analytical method.

Analytical methods can still be strengthened by improving both extraction efficiencies and chromatographic detection. Further development of monoclonal antibodies in preliminary clean-up will help in HPLC methods for many commodities that contain many interfering substances. Advances in mobile phases, columns and detectors will probably allow routine aflatoxin determinations at the picogram level. New extraction techniques such as supercritical fluid extraction and detection methods need to be developed to eliminate the need for fluorescence enhancement and produce lower costs, increased efficiency, improved safety and meeting waste disposal requirements. Future methods for aflatoxin determination will certainly be more sensitive and improvements made in sampling will make aflatoxin control easier.

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Review

Chromatographic methods for the determination of pesticides in foods

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ABSTRACT

Chromatography is the most important technique available to the analyst dealing with the determination of pesticide residues in food, feed and environmental samples. Numerous methods for pesticide residues in foods have been developed in the past few years, and this paper reviews some of the most important procedures. A great variety of chromatographic methods, such as solid-phase extractions, column chromatographic clean-up methods, thin-layer, gas, high-performance liquid and supercritical fluid chromatography, and their coupling with sensitive and selective detection methods are surveyed.

CONTENTS

1. INTRODUCTION

In modern agricultural food production, good results can be obtained only with the help of pesticides, but these materials contaminate the environment and some more or less persistent pesticide residues remain on the surface of or inside the products. Methods for pesticide residue determinations for the qualification of food products are under rapid development. The residue limits given by the World Health Organization are becoming even lower and lower creating an ever increasing demand for more selective and sensitive methods. The great variety of pesticides need multi-residue methods but their applicability is limited by the different nature of the food materials. These multi-residue methods allow the simultaneous determination of herbicides, insecticides, fungicides and others in one extract. As the concentration of the pesticide residues in food material is in the nanogram, picogram or sometimes femtogram per gram range, their evaluation can be carried out only by extremely selective and sensitive detection methods.

The determination of pesticide residues in food

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materials consists in sample preparation and the determination. The role of chromatography is very important in both of these steps. Chromatography can be used as a preliminary concentrating procedure which can be continued with column chromatography as a part of the clean-up. Moreover, chromatography also plays a very important role in the determination step. Gas (GC), supercritical fluid (SFC), high-performance liquid (HPLC), thin-layer (TLC) and overpressure thin-layer chromatography (OPTLC) and combinations of these methods with other techniques, *e.g.*, mass spectrometry (GC–MS, HPLC–MS), will be discussed here.

2. SAMPLE PREPARATION: EXTRACTION AND CLEAN-UP

The sample preparation consists of the extraction and clean-up steps, which are influenced by the final determination. If the determination is selective enough, the clean-up need not be so thorough, but less selective detection methods need more efficient clean-up steps.

Large numbers of methods have been developed for the determination of pesticides in different food materials. These multi-residue methods offer the possibility of the detection and determination of organochlorine (OC), organophosphate (OP) and carbamate insecticides, triazine and thiocarbamate herbicides, dithiocarbamates and other fungicides and other contaminants, sometimes in one extract.

The method of the Association of Official Analytical Chemists (AOAC) [1] is representative of the internationally recognized multi-residue methods that allow the determination of numerous OC and OP insecticides, carbamates and other pesticides in fatty and non-fatty food samples and in foods with high or low sugar contents, etc. According to this method, extraction is carried out with acetonitrile, followed by liquid–liquid partition and Florisil column clean-up when OC insecticides are to be determined in fatty foods and sweep co-distillation for OP insecticides. The AOAC multi-residue method was extended to the determination of thiocarbamate herbicides in food samples of plant origin [2]. In several samples of maize products (Table 1) some co-extracts were present, which disturbed the determination. Interferences could be eliminated by further clean-up, steps, *e.g.*, coagulation with ammonium chloride and urea solutions followed by Florisil column chromatography (Fig. 1).

Several other methods use sweep co-distillation [3,4] in the determination of OP insecticides. Size-exclusion chromatography [5–7] and column chromatography on charcoal and mixed columns [8–10] have also been used as clean-up methods in OP determinations. Recently published methods use disposable cartridges. For example, an Extrelut-20 cartridge was applied for the clean-up of fruit and vegetable extracts containing eighteen OP insecticides (Table 1) [11]. OP insecticides were determined in tea extracts, a sulphuric acid treatment being necessary before the Florisil column clean-up (Table 1) [12]. Twenty-three persistent OC insecticides were investigated in lipid-rich food samples. Negative interfering peaks were observable in the gas chromatograms when Florisil column clean-up was used alone. These interferences were eliminated with a sulphuric acid treatment on a solid matrix column (Table 1) [13].

A Carbopack B cartridge and sulphonic acid-type silica-based cation-exchange (SCX) columns were applied for the clean-up of triazine-containing vegetable extracts. HPLC determination gave the recovery data shown in Table 1 [14].

Phenylurea herbicides were studied in the presence of aniline-type compounds on a graphitized carbon black (Carbopack B) cartridge connected with a strong cation exchanger. This method was compared with the use of a C_{18}-bonded silica cartridge. The recoveries were established by reversed-phase (RP) HPLC with UV detection (Table 1) [15]. Better recoveries were reported using a C_{18}-bonded silica cartridge compared with Carbopack B in the determination of 24 basic–neutral and eleven acidic pesticides in water samples (Table 1) [16] by RP-HPLC. The extraction is seven times quicker than that with Carbopack B because it does not need any pH adjustment. Nevertheless, Carbopack B seems to be more adaptable in field use.

Rapid and selective on-column extraction of OC pesticide residues from milk samples was carried out. The extraction system minimizes the fatty co-extractives and gives almost quantitative recoveries of pesticides (Table 1) [17].

TABLE 1

EXTRACTION AND CLEAN-UP PROCEDURES

Compound	Sample	Extraction	Clean-up	Recovery (%)	Method of analysis	Ref.
EPTC Butylate Molinate Lindane *p,p'*-DDT	Deep-frozen peas Potatoes Beans	Acetonitrile	Florisil	73–100	TLC densitometry	2
p,p'-DDE Trichlorphon Dimethoate Mevinphos Methylparathion	Maize Maize flour Maize grits Shelled grain		Coagulation Florisil			
Chlorpyrifos metabolites	Banana pulp	Acetone	Silica gel (15% water) Charcoal–MgO–Celite (1:2:4)		TLC	9
OC, OP Carbamates Triazines	Apple Spinach Carrot	Acetone	Charcoal–magnesia–diatomaceous earth (1:2:4)		TLC	10
Methacriphos Fonofos Fenchlorphos Dimethoate Parathion-methyl Parathion Metidathion Diazinon Ten other OP	Broccoli Cauliflower Onion Radish Peach Tomato	Acetone	Extrelut-20 CH_2Cl_2–light petroleum (1:3)	75–107	GLC	11
Malathion Fenitrothion Quinalphos Dimethoat	Tea	Toluene–MeOH (3:1)	Sulphuric acid Florisil Toluene–acetone (98:2)	96–97	GLC 6% OV-101 Chromosorb W (80–100 mesh), NPD	12
23 Persistent OC	Human milk Cow milk Vegetable oil		Florisil Extrelut-1 Sulphuric acid	89–117 (49)	OV-17 + QF-1 (1.5% + 1.95%) Chromosorb W, ECD	13
Simazine Simetryne Atrazine Prometon Ametryn Propazine Prometryn	Water Vegetables Lettuce Spinach Chicory Endive Kale	CH_3CN–H_2O (6:4)	Carbopack B CH_2Cl_2–MeOH (6:4) (a) CH_2Cl_2–MeOH (6:4) (b) CH_2Cl_2 (c) CH_2Cl_2–CH_3CN (6:4)	 65–100 82–86 96–98	RP-HPLC, UV LC-18-DB	14
17 Triazines and carbamates	Water		(a) Carbopack B Amberlite CG-120-I (b) C_{18}-bonded silica	92 6.3–69.6	RP-HPLC LC-18	15

(Continued on p. 356)

TABLE I *(continued)*

Compound	Sample	Extraction	Clean-up	Recovery (%)	Method of analysis	Ref.
(a) 24 Basic–neutral pesticides	Drinking water		Carbopack B	95	RP-HPLC LC-18	16
(b) 11 Acidic pesticides			C_{18}-bonded silica	76		
HCB, α-HCH β-HCH Endrin Heptachlor epoxide Dieldrin *p,p'*-DDE *p,p'*-DDT	Milk Milk powder	Light petroleum–CH_3CN–EtOH (100:25:5)	Chem-Elut CE 1010 Florisil	77 94–113	GLC, ECD OV-17 + QF-1 (1.5% + 1.95%) Chromosorb W HP	17

3. METHODS FOR THE DETERMINATION OF PESTICIDE RESIDUES

3.1. Thin-layer chromatography

For the determination of pesticides in food and feed samples, at least two independent methods are necessary to decide whether the sample is appropriate for consumption or not. Pesticide-like interfering co-extractives can cause serious problems. In ambiguous cases alternative methods are applied. TLC can serve as one of the alternative methods when the qualitative results are quantified by *in situ* densitometry. The detection methods applied should have two orders of magnitude higher sensitivity than the given residue limit of the compound being studied.

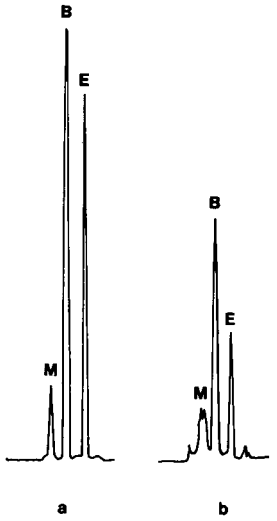


Fig. 1. Densitogram of thiocarbamates in deep-frozen beans by AOAC method [2]: (a) with coagulation; (b) without coagulation. E = EPTC; B = butylate; M = molinate.

OP, OC and carbamate-type compounds were investigated by the extended AOAC method for the determination of thiocarbamates. Possible interferences were checked. The detection of OP compounds was carried out with the chromogenic agent 4-(4'-nitrobenzyl)pyridine (NBP), that of OC compounds with silver nitrate–2-phenoxyethanol and that of thiocarbamates with 2,6-dibromobenzoquinone N-chloroimine (DBI) and N,2,6-trichlorobenzoquinone N-chloroimine (TBI) in acidic solution (Table 2) [2,18]. Possible interferences were investigated. The sensitivity of these detection systems was studied on different TLC supports. Reversed-phase TLC on alumina G and adsorption chromatography on the same support was compared with the use of Polygram Cel 300. The latter gave the most sensitive reaction (0.05 μg per spot) in OC detection. Only the halogen-containing chlorfenvinphos gave a positive reaction; the other OP and thiocarbamates did not react. In analyses for OP compounds, sensitive detection was achieved on Silufol and Polygram SIL G, plates with 0.05 μg per spot sensitivity. The chromogenic reagents DBI and TBI detected not only the thiocarbamates but also some other sulphur-containing OP compounds with 0.02–0.05 μg per spot sensitivity. These detection methods were also checked on different supports such as Kieselgel 60,

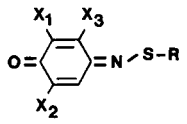
TABLE 2

THIN-LAYER CHROMATOGRAPHY OF PESTICIDES

Compound	Sample	TLC			Recovery (%)	Ref.
		Plate	Eluent	Detection		
EPTC Butylate Molinate Pebulate Cycloate	Potatoes Beans Peas Maize Flour	Silufol Polygram Sil G	Hexane–diethyl ether–acetone (7.5:2:0.5)	DBI TBI in CH_3COOH	80–100	2
p,p'-DDT *p,p'*-DDE	Grits Grains	RP alumina G	CH_3CN–acetone–MeOH–H_2O (40:18:40:2)	$AgNO_3$–2-phenoxy ethanol		
		Alumina G Polygram Cel 300				
Mevinphos Dimethoate Fenthion Chlorfenvinphos Trichlorphon		Silufol Polygram Sil G Kieselgel 60 Silica gel 60–Kieselguhr	Hexane–diethyl ether–acetone (7.5:2:0.5) Hexane–diethyl ether (8:2)	NBP		
Carbofuran	Goose and pig feed	Silica gel G	CCl_4–EtOH–acetone (4:1:1)	TBI in NaOH solution		21
Herbicides Insecticides Fungicides Phenylureas Modified ureas Carbamates N-Heterocyclic compounds	Drinking water		Automated multiple development		70–120	22
Dursban Metathion Dimethoate Phosalone	Apple	Silica gel G	Hexane–acetone (9:2)	Indoxyl acetate		24
Insecticides Acaricides Fungicides	Fresh apple Processed apple	Two-dimensional TLC Silica gel GF_{254}	(1) Cyclohexane–acetone (10:1) (2) Light petroleum–benzene–ethanol (65:30:5)	UV 255, 366 nm Bromophenol blue		25

silica gel 60–Kieselguhr and Silufol plates [2]. The best and most reproducible results were obtained on Silufol plates (Table 2).

The chemical processes responsible for the detection of the thiocarbamate herbicides were also studied. The detection of thiocarbamate herbicides was carried out in acidic solutions, giving yellow spots on a white background. With DBI some side-reactions were taking place, a higher halogen content being observable in the mass spectrum of the coloured compound. Surprisingly, chlorine was also present in the product formed in the reaction of DBI. Here 2,6-dihalo- and 2,3,6-trihalobenzoquinone imine derivatives were observed (Fig. 2). These groups of the mixed halogenated benzoquinone derivatives were separated by flash chromatography using xylene or mesitylene as eluents. Here some charge-transfer complex formation is assumed. The 2,6-dihalobenzoquinone imine derivatives, having very small differences in their polarity, were separated by OPTLC using cyclohexene as mobile phase [19]. In the TLC detection of thiocarbamate herbicides,



X_1 = Br, Cl
X_2 = Br, Cl
X_3 = H, Br, Cl
R = alkyl chain

Fig. 2. Coloured compounds formed in the TLC detection of thiocarbamates with TBI and DBI.

DBI gave more sensitive detection but TBI gave more reproducible results. It should be mentioned that oxidative metabolites of S-ethyl dipropylthiocarbamate (EPTC), butylate and other thiocarbamates also gave positive reactions with TBI and DBI [20]. Some sulphur-containing pesticides and thio- and dithiophosphates gave positive reactions with good sensitivity [2]. This method was developed for the *in situ* densitometric evaluation of thiocarbamates and other pesticide in food extracts (Fig. 1).

The same reagents can also be used for carbofuran detection in alkaline solution. Contaminated goose and pig feeds were analysed after dichloromethane extraction. The residues were reacted with an alkaline solution of TBI and the blue indophenol derivative obtained was separated on silica gel G plates (Table 2) [21].

A straightforward method for drinking water analysis using high-performance TLC was published. Numerous pesticides (Table 2) were extracted by solid-phase extraction on a C_{18}-modified silica cartridge. A correct pH adjustment helped the separation of the pesticides. Automated multiple development (AMD) was applied in TLC. The limitation of the method is the adjustment of the pH during the elution. The TLC results should be checked by GC or HPLC [22].

Another extremely specific detection method was developed for endosulfan and phosphamidon residues. The plates were sprayed with cobalt acetate in alkaline solution and subsequently with tolidine in acidic media. Neither OC insecticides (endrin, aldrin, dieldrin, DDT), OP insecticides (malathion, parathion, dimethoate, quinalphos, phorate, fenitrothion) nor carabamate insecticides (baygon, carbaryl and carbofuran) gave any coloured spots. Further, no reaction was reported with amino acids, peptides and proteins present as co-extractives. This reagent is five times more sensitive than ethanolic diphenylamine and *o*-tolidine or *o*-dianisidine with UV irradiation [23].

Some sulphur-containing OP insecticides in apples were determined by GC and TLC determination. Indoxyl acetate was applied as developer (Table 2) [24]. Insecticides, acaricides and fungicides were determined by TLC in fresh and processed apples. After two-dimensional TLC separation, eighteen pesticides were detected by UV irradiation and spraying with bromophenol blue solution (Table 2) [25].

The enzyme inhibition method gives the most sensitive detection for pesticides on TLC plates. These reactions are very sensitive and selective. A recently published review deals with the enzyme inhibition method using selected enzymes and substrates in different combinations [26].

Pesticide detection by enzyme inhibition can be thought of as fluorescent detection of the compounds. After hydrolysis, the enzyme converts the substrate into a fluorescent derivative so the background becomes fluorescent and the inhibition spots of the pesticides develop. Changing the substrates and the esterases can enhance the sensitivity. Some enzymes, such as acetylcholine esterases in pig liver, human plasma, horse serum and beef liver, with substrates such as indoxyl acetate, 5-bromoindoxyl acetate and butyrylthiocholine can give very sensitive and selective detection with nanogram per spot or higher sensitivity. OP insecticides and carbamate-type compounds were detected by this method [18].

Some other biological detection methods should be mentioned here. Inhibition of the Hill reaction is useful in the detection of photosynthesis-inhibiting herbicides. This procedure gives as high a sensitivity for herbicide detection as the acetylcholine esterase-inhibiting insecticide tests [18].

Some fungicide tests were published earlier which offer very sensitive detection for compounds having fungicidal activity. *Culvularia lunata, Phytium ultimum, Cladosporium cucumerium* and numerous other fungal strains were applied in these tests [18].

Pesticides can be revealed by liquid crystals according to recently published results. The developed TLC plates were covered with a porous foil which was impregnated with liquid crystals. When the foil was pressed on to the plate, the presence of

pesticides disturbed the structure of the liquid crystals in the foil. This caused a change in the light transmittance of the layer, permitting the determination of the spot areas [27].

3.2. Gas chromatography

The GC analysis of pesticides is of great importance nowadays. Numerous methods have been developed for large numbers of insecticides, fungicides, herbicides, etc., showing very different chemical behaviour [28–30].

Some of the interesting capillary GC (cGC) methods will be discussed here. The earlier recommended AOAC method for the extraction of pesticide residues from non-fatty foods was based on an extraction with acetonitrile or acetonitrile–water. It was modified in 1985 by an acetone extraction. This was given as an official final action method in 1986 [30]. The efficiencies of these methods were checked by cGC with different detection modes. In fruit samples OP and OC insecticides were extracted by two different methods and determined by cGC using different detectors. The results were checked by GC–MS (Table 3) [31]. Lemon essential oil extracts were obtained either by pressure extraction of the peel half-cups or by excoriation of the whole fruit using an on-line extraction system (Table 3) [32]. According to another method, nine halogen-containing pesticides were determined on a dimethylpolysiloxane-coated capillary column with electron-capture detection (ECD) after extraction and a short-column Florisil clean-up (Table 3) [33]. Rice and soybean samples were studied by cGC in point of the determination of the α-BHC and carbaryl content with ECD and nitrogen–phosphorus-specific detection (NPD) (Table 3) [34]. About 20 pesticides were investigated after three successive extractions in fruit and vegetable products from Spain (Table 3) [35].

TABLE 3

GAS CHROMATOGRAPHIC DETERMINATION OF PESTICIDES

Compound	Sample	Extraction	Clean-up	Recovery (%)	Detection	Determination	Ref.
Dimethoate Lindane Fenitrothion Malathion Chlorpyrifos Methidathion Tetradifon Tetradithion Phenthoate	Fruit	(1) CH_3CN–H_2O (2) Acetone (3) Acetone–MeOH (4) CH_3CN, Na_2SO_4	Partition Sep-Pak C_{18}	(1) 82–140 (2) 87–129 (3) 81–130 (4) 81–129	ECD NPD FPD GC–MS	Chromosorb W SPB-5 cGC	31
Parathion-methyl Parathion-ethyl Methidathion Quinalphos Diazinon Fenitrothion Malathion Bromophos-ethyl	Lemon oil	Pressure extraction On-line extraction system			NPD	cGC	32
Chorpyrifos Dichlofluanid Dichloran Endosulfan γ-HCH Procymidon Vinclozolin	Pepper Cucumber	EtOAc Na_2SO_4	Florisil	>80	ECD	Dimethylpolysiloxane cGC	33

(Continued on p. 360)

TABLE 3 *(continued)*

Compound	Sample	Extraction	Clean-up	Recovery (%)	Detection	Determination	Ref.
α-BHC Carbaryl	Rice Soy bean	Acetone–MeOH	Bio-Beads S-X_3 Cyclohexylamine–CH_2Cl_2 (1:1)	>83 >81	ECD NPD	BP-1 cGC	34
19 Pesticides	Melon Sweet pepper Cucumbers Lettuce Zucchini					HP-17 BP-1 SPB-1	35
C-, P-. Cl-, F-, N-, S-containing pesticides	12 Agricultural commodities		No clean-up		AES	cGC	36
Cl-, F-, P-containing pesticides					ECD, ED, NPD, FPD		
Bromoxynil	Onion	80% EtOH NaOH, hydrolysis	NaCl satd. soln. Diethyl ether Florisil	94–117	Derivatization CH_2N_2	HP-1 cGC	37
Triadimenol	Fruit Cereal	Acetylation	Florisil Light petroleum–diethyl ether (96:4) Light petroleum–EtOAc (3:2)	83–96	TID	5% OV-101 Chromosorb W HP	38
Paraquat Diquat	Potatoes Rapeseed	Deriv. $NaBH_4$	Partition	86–100	NPD GC–MS	5% Apiezon L 3% KOH on Interton Super	39
Ethoprop	Mint hay Oil Spearmint Peppermint	Hexane	Charcoal Florisil		GC GC–MS	7% OV-17 Chromosorb W HP DB-5 capillary	40
Carbaryl Captan Dichloran Dimethoate Methamidophos Phosmet	Apple Peach Tomato Potato			73–120	GC–CI-MS	DB-1 Methyl fused silica	41
Dicamba	Dried tobacco	Hexane–diethyl ether (1:1)	Derivatization		On-line LC–GC UV ECD	LC Spherisorb S-5-W OV-16-OH ID fused silica	45
OC	Butter	Melting Na_2SO_4			LC–GC ECD	Biol-Sil ODS-10 Capillary SE-52	46

The applicability of detectors were compared with atomic emission spectrometric (AES) detection in the GC of twelve agricultural products. The extracts were prepared according to the procedure of the California Department of Food and Agriculture and no clean-up was used. AES was used in the C-, P-, Cl-, N- and S-selective modes and showed higher selectivity in the determination of chlorine-, fluorine- and phosphorus-containing pesticides than other detection methods (Table 3) [36].

The selectivity of determination can be enhanced by derivatization. An extract of bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) was first hydrolysed and subsequently converted with diazomethane into its methylated form. The derivative-containing extract was cleaned up on a Florisil column and determined by cGC (Table 3) [37].

The fungicides triadimenol [1-(4-chlorophenoxy)-3, 3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] and bitertanol [1-(biphenyl-4-yloxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] can be determined in acetylated form after extraction. The derivative-containing sample was passed through a Florisil column and determined by GC (Table 3) [38].

Diquat and paraquat are rapid-acting herbicides. These compounds were determined in potatoes and rapeseed after conversion into their volatile derivatives by hydrogenation with sodium tetrahydroborate. Another possibility is the dequaternization of these bipyridinium herbicides by pyrolysis. This approach was followed in GC–MS studies of biological samples (Table 3) [39].

Ethoprop was determined in mint hay and oil by GC. Ethoprop residues were present in the oil samples in 100-fold higher concentrations than in the mint hay according to GC–MS studies (Table 3) [40]. Carbamate and OP residues were investigated by cGC–MS method. Twelve pesticides and two metabolites were determined in 25 different samples each of four different food materials. A computer program allowed a search for several hundred target ions. This cGC–CI-MS methodology can be applied with convenience in routine analyses by regulatory agencies (Table 3) [41]. OP insecticides were measured in biological samples without any interferences [42]. A review on monitoring pesticides in food, feed and environmental samples has been published [43].

The first example of coupled LC–GC in pesticides determinations was reported for atrazine in 1987 [44]. More recently dicamba was studied in dry tobacco samples by on-line coupled HPLC–GC. In this procedure no liquid–liquid partitioning is necessary. After simple extraction, the dicamba residue is converted with diazomethane into its ester. Normal-phase HPLC serves for clean-up and the final determination is carried out by GC. The main problem here is the concurrent evaporation of the eluent during transfer of the extract between the two systems. This problem was overcome as reported (Table 3) [45]. OC pesticides were determined in fat samples by this on-line HPLC–GC method (Table 3) [46].

Another technique was reported for the determination of 30 polychlorinated biphenyl congeners using two capillary columns with non-linear multilevel calibration. The capillary columns, which were operating with parallel coupling, exhibited different polarities. Various seal extracts were analysed in this way [47].

3.3. Supercritical fluid chromatography

The SFC method is very useful when GC and HPLC are inappropriate [48]. In addition, more effective separations can be achieved with SFC than with GC from the point of view of the number of effective plates or separation speed. Modified mobile phases used as supercritical fluids and packed or capillary columns give a choice for separations of pesticides of different natures. The best detection method is MS, which has high selectivity and sensitivity. The determination of less volatile analytes, *e.g.*, labile insecticides and herbicides, is the most interesting application of SFC–MS. Some examples of this rapidly developing method used in pesticide analysis will be discussed here.

A splitless injection method was developed for interfacing microbore or high flow-rate capillary SFC with MS detection [48]. Eight pesticides were examined on a microbore column with carbon dioxide elution. The polar pesticides could be separated with much better efficiencies when 1% of methanol was added to the carbon dioxide mobile phase. Peak tailing was eliminated and retention was also reduced (Table 4) [48].

A high flow-rate interface for SFC–MS determination of OP insecticides has been published recently. This interface allows a pressure-programmed

separation on microbore HPLC columns. For analysis for thermally unstable compounds of high molecular mass and low volatility, a lower temperature separation method was necessary. SFC–CI-MS was very useful in the determination of eight OP insecticides. These compounds were determind on an amino-phase microbore column. Better resolution was obtained when 2% (v/v) of 2-propanol was added to the carbon dioxide mobile phase on the column mentioned above. A C_{18}-bonded non-polar phase column gave less effective separations of these OP compounds under the same analytical conditions, *e.g.*, pressure programming and addition of 2-propanol to the mobile phase (Table 4) [49].

Eight non-volatile triazine and triazole herbicides were also investigated. A modified HPLC system with UV detection was used, eluting first with carbon dioxide alone, but it was found that a much better separation was achieved with gradient elution using carbon dioxide containing 2.4–33% of methanol (Table 4) [50].

Packed capillary SFC of OP insecticides with phosphorus-selective detection was reported recently. The carbon dioxide eluent was modified with methanol or 2-propanol, leading to the determination of the OP insecticides in onion and tomato samples. Linearity was obtained over a four orders of magnitude range (Table 4) [51].

Several thermally labile pesticides (ureas and OP compounds) were measured by capillary column SFC without any modification of the mobile phase using ECD. Picograms and high femtograms were given as detection limits for nitro- and halogen-containing pesticides, respectively [52].

3.4. High-performance liquid chromatography

HPLC is suitable for compounds having low volatility or those which are thermally unstable. HPLC is just as important as GC in pesticide residue analyses. Some recently published methods for analyses for fungicides, herbicides, insecticides and growth regulators are summarized here.

A method was published for the determination of four fungicides in must and wine samples. The

TABLE 4

SUPERCRITICAL FLUID CHROMATOGRAPHY OF PESTICIDES

Compound	Sample	SFC conditions	Column	Detection	Ref.
Alachlor BMPC Propachlor Propoxur Linuron Carbofuran Carbaryl Diuron		CO_2 CO_2–MeOH (1%) 50–100°C 400–450 bar	Microbore C_{18} silica Capillary (SE-54)	SFC–CI-MS	48
Chlorpyriphos-methyl Chlorpyriphos Iodofenphos Leptophos Methidathion Tetrachlorvinphos Phosmet Famphur		CO_2, 75°C, 410 bar 25 bar/min. 2% 2-propanol–CO_2	Amino phase Microbore	SFC–MS	49
Triazine Triazole Herbicides	Cherries	CO_2–MeOH, gradient elution (2–33%)	Deltabond Cyanopropyl	SFC UV	50
Phoxim Dimethoate Azinphos methyl	Onion Tomato	CO_2–2-propanol CO_2–MeOH	Packed Capillary	SFC TID	51

degradation process of these fungicides was followed during the winification process. Sample preparation is very simple: the must and wine samples are extracted with benzene and the organic layer is evaporated to dryness, the residues being analysed by RP-HPLC. Water–methanol eluents gave reproducible results when buffer was applied; an acetonitrile–water gradient system gave reproducible results without buffers. During the alcoholic fermentation, carbendazim and metalaxil levels decreased. In red wine extracts interfering co-extractives were observed (Table 5) [53].

A multi-residue method for fungicides in fruit and vegetables involving solid-phase extraction (SPE) was published. Fungicides eluted from an SPE cartridge were determined by GC or HPLC. The rise in the baseline was due to captafol decomposition under GC conditions. More reproducible results were obtained when determination was carried out by HPLC. UV detection was affected by some interferences but fluorimetric detection gave reproducible results for several samples (Table 5) [54].

Bensulfuron-methyl residues in rice grain and straw samples were investigated by HPLC with photoconductivity detection. Different sample preparation methods were published for grain and straw samples (Table 5) [55]. The qualification of feed samples is equally important. A method was published for hexazinon and monuron residues in alfalfa tissues. The recovery was checked after each sample preparation step. The best recoveries were obtained according to the method given in Table 5. For HPLC analysis a precolumn was also applied [56].

Cyanazine and bentazone herbicide residues in sugar maize and surface water were examined using HPLC and on-line clean-up column switching (Table 5) [57]. Phenoxy acid herbicides were determined in water samples. The compounds studied were converted with 9-anthryldiazomethane into their derivatives, which were detected with a fluorescence detector. The recoveries were >95% (Table 5) [58].

Some recently published HPLC methods deal with the analysis of insecticides in food samples. Carbamate (carbaryl) in fruit juice samples can be determined by HPLC. Residues were collected on an SPE cartridge (Table 5). However, the fruit matrix does not allow detection limits for carbaryl as low as in water, being at the low-ppb (parts per 10^9) level [59].

Flufenoxuron, a slow-acting growth regulator, was determined in apples and kiwi fruit by HPLC. Residue recoveries were 81–117% (Table 5) [60]. Ethiofencarb, a systemic insecticide, and its oxidative metabolites (sulphoxide and sulphone) were identified in lettuce by HPLC with 90–103.1% recoveries. The method is simple and does not need any derivatization [61].

HPLC combined with thermospray mass spectrometry (TSP-LC–MS) in the positive- (PI) and negative-ion (NI) modes was used for the determination of six pesticides and their photodegradation products in water samples. The pesticides were representatives of carbamates, chlorotriazines, phenylureas and OP compounds. A combination of the PI and NI modes allows the identification of numerous photodegradation products (Table 5) for different pesticides. Some of the degradation products could be detected only in the PI or NI mode, so for precise information on toxic metabolites or degradation products both detection methods should be used (Table 5) [62].

4. CONCLUSIONS

Recently published methods for the determination of pesticide residues have been reviewed. The most important chromatographic sample preparation and determination methods have been discussed. Newer chromatographic methods applied in pesticide residue determinations such as solid-phase extraction and supercritical fluid chromatography are becoming increasingly important. Gas chromatography and high-performance liquid chromatography provide the basis of numerous determination methods alone or in combination with very sensitive and selective detection methods such as mass spectrometry. Thin-layer chromatography combined with densitometry is also applied as an alternative method because of its simplicity.

5. ABBREVIATIONS

GC	Gas chromatography
cGC	Capillary gas chromatography
SPE	Solid-phase extraction
HPLC	High-performance liquid chromatography
TLC	Thin-layer chromatography

TABLE 5

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PESTICIDES

Compound	Sample	Extraction	Clean-up	HPLC column	Eluent	Detection	Ref.
Carbendazim Metalaxyl Folpet Propiconazole	Must Wine	Benzene	No clean-up	Spherisorb ODS-1 (C_{18})	CH_3CN–H_2O gradient	UV	53
Dichloram Chlorothalonil Vinclozolin Triadimefon Anilazine Captan Folpet Procymidone Iprodion *o*-Phenylphenol Biphenyl	Grape Apple Tomato Pear Cucumber Strawberry Orange Potato	Acetone SPE (C_{18}) (Supelco)	MeOH–H_2O (40:60)	Bondapak (C_{18})	MeOH–buffer (60:40)	Fluorimetric	54
Bensulfuron-methyl	Rice Grain	CH_2Cl_2	C_{18} Bond Elut (SPE)	Zorbax Sil	Hexane–isopropanol–MeOH–CH_3CN–CH_3COOH–H_2O (750:125:100:25:2:1)	Photoconductivity	55
	Rice straw	0.5% CH_3COOH CH_2Cl_2	Several partition steps		As above (720:110:110:55:2:1)		
Hexazinone (monuron)	Freeze-dried alfalfa	H_2O–MeOH	Hexane, $CHCl_3$	Precolumn CO:Pell ODS-18 R-Sil C_{18}	MeOH–H_2O (1:1)	UV, 254 nm	56
Bentazon Cyanazine Two metabolites	Sugar Maize Surface water	CH_2Cl_2 6 *M* HCl CH_2Cl_2–CH_3CN		RP-18 Hypersil ODS	MeOH Phosphate buffer	UV, 299 nm	57

2,4-D MCPA MCPP MCPB	Water	Hexane–EtOAc (20:80)	Silica gel Hexane–EtOAc (95:5), derivatization	RP TSK-gel ODS-120T	CH_3CN–H_2O–THF (72:25:3)	Fluorescence	58
Carbaryl	Apple Cherries Pineapple Orange Banana Cranberry Grape Juice, etc.	C_{18} Sep-Pak CH_3CN–H_2O		Ultramex C_{18}	MeOH–H_2O–CH_3CN (40:45:15)	UV, 224 nm	59
Flufenoxuron	Fresh fruit Apple Kiwi fruit	MeOH–H_2O	MeOH–hexane	Zorbax ODS HPLC RP-8	CH_3CN–H_2O (74:26)	UV, 254 nm	60
Ethiofencarb Ethiofencarb sulphoxide Ethiofencarb sulphone	Lettuce	CH_2Cl_2		RP-18 C_8–H	H_2O–CH_3CN (1:1), (6:3) (7:3), (6.5:3.5)	UV, 190 nm	61
Aldicarb Carbaryl Linuron Fenitrothion Cyanazine	Water			LiChrospher 100 RP-18 Spherisorb ODS Polyglosil 500-7	MeOH–H_2O (1:1) + 0.05 *M* ammonium acetate MeOH–H_2O (7:3) + 0.05 *M* ammonium formate	TSP-LC–MS, PI, NI	62
Parathion-methyl				C_{18}	CH_3CN–H_2O (1:1) + 0.05 *M* ammonium acetate		

OPTLC	Overpressure thin-layer chromatography
AES	Atomic emission spectrometric detection
AMD	Automated multiple development
ECD	Electron-capture detection
ED	Electrochemical detection
NPD	Nitrogen–phosphorus-selective detection
FPD	Flame photometric detection
TID	Thermionic detection
CI-MS	Chemical ionization mass spectrometry
EI-MS	Electron impact mass spectrometry
TSP-LC–MS	Thermospray-liquid chromatography–mass spectrometry
PI	Positive ion mode
NI	Negative ion mode
OC	Organochlorine
OP	Organophosphate
DBI	2,6-Dibromobenzoquinone N-chloroimine
TBI	N,2,6-Trichlorobenzoquinone N-chloroimine
NBP	4-(4′-Nitrobenzyl)pyridine
EtOAc	Ethyl acetate
EtOH	Ethanol
MeOH	Methanol
THF	Tetrahydrofuran

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Review

Chromatographic separation of cholesterol in foods

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ABSTRACT

Based on the current literature and on experience gained in the laboratory, a simplified procedure using direct saponification (0.4 *M* potassium hydroxide in ethanol and heating at 60°C for 1 h) is the most appropriate method for the determination of total cholesterol in foods. Extraction of the unsaponifiable matter with hexane is efficient and no extra clean-up is required before quantification. An internal standard, 5α-cholestane or epicoprostanol, should be added to the sample prior to saponification and, together with reference standards, carried through the entire procedure to ensure accurate results. A significant improvement in cholesterol methodology has been achieved by decreasing the sample size and performing all the sample preparation steps in a single tube. The method has the advantages of elimination of an initial solvent extraction for total lipids and errors resulting from multiple extractions, transfers, filtration and wash steps after saponification. The resulting hexane extract, which contains a variety of sterols and fat soluble vitamins, requires an efficient capillary column for complete resolution of cholesterol from the other compounds present. The development of fused-silica capillary columns using cross-linked and bonded liquid phases has provided high thermal stability, inertness and separation efficiency and, together with automated cold on-column gas chromatographic injection systems, has resulted in reproducible cholesterol determinations in either underivatized or derivatized form. If free cholesterol and its esters need to be determined separately, they are initially extracted with other lipids with chloroform–methanol followed by their separation by column or thin-layer chromatography and subsequently analysed by gas or liquid chromatography. Although capillary gas chromatography offers superior efficiency in separation, the inherent benefits of liquid chromatography makes it a potential alternative. Isotope dilution mass spectrometry has been widely accepted as a reliable analytical method for highly accurate determination of cholesterol in serum and several definitive methods have been reported. The combination of capillary gas chromatography with mass spectrometry has become an excellent approach for the determination of cholesterol in complex mixtures of sterols and tocopherols, providing high resolution with positive identification. When used to determine cholesterol in multi-component foods, spectrophotometric methods have been documented to overestimate significantly the amount of cholesterol owing to the presence of other interfering substances. A re-evaluation of food products should be undertaken using the more specific chromatographic methods to accumulate data that will more accurately reflect the true cholesterol content.

CONTENTS

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1. INTRODUCTION

Cholesterol is a 27-carbon steroid which is present in all animal tissues as a major structural component of cellular membranes. It is the precursor of bile acids, provitamin D_3 and the steroid hormones. Cholesterol can be present in the free form or esterified at the hydroxyl group with fatty acids of various chain length and saturation.

Public interest in cholesterol has increased owing to awareness and publicity of the relationship of plasma cholesterol levels to the risk of developing coronary artery disease. The relationship between dietary cholesterol and heart disease has been the subject of much research. Owing to public concern, specific recommendations have been made regarding desired changes in the diet [1] and as a result there is a need for more complete labelling for the levels of cholesterol in various food products.

Major sources of cholesterol in the diet are animal products, including eggs, milk and meat. Fish is relatively rich in cholesterol, but in shellfish (*e.g.*, clams, oysters and scallops) other sterols are present in substantial amounts [2,3]. Much of the earlier data on the cholesterol content of food was produced using either spectrophotometric or gravimetric methods of analysis [4]. These data showed a wide variation in the cholesterol content; values often differed among investigators by more than 100%. Various spectrophotometric [5,6] and enzymatic [7] methods, originally developed to determine cholesterol in blood, were found to be unsuitable for the determination of cholesterol in food. Owing to various interferences caused by the presence of a considerable amount of plant or other sterols in food, these methods normally gave higher values for cholesterol content than the newer chromatographic procedures [3,8–11]. Problems with interferences and limitations of spectrophotometric methods have been discussed [4,8,12]. Differences in the cholesterol contents of various foods as reported in the literature, therefore, can be attributed to a great extent to differences in the analytical procedures used. Chromatographic procedures are highly specific and therefore more precise and accurate for the determination of cholesterol in food. These procedures also have the benefit of allowing the simultaneous measurement of plant sterols. Gas chromatographic (GC) procedures have been accepted as the method of choice for the determination of cholesterol in food [4,10,13,14]. However, even among the chromatographic techniques used there is a lack of standardization of analytical conditions which could lead to inaccurate data. This review is concerned with potential problems in analytical procedures associated with sample preparation and in particular with the chromatographic methods used in the determination of cholesterol in food.

2. SAMPLE PREPARATION

2.1. Lipid extraction

The method of sample preparation can have a significant influence on the results of cholesterol determination. Extraction of total lipids with a solvent has usually been the first step in sample preparation. Numerous extraction procedures involving many different solvent systems have been applied to foods (Table 1). Previous methods for the determination of fatty acids using 4 *M* HCl digestion followed by diethyl ether extraction were found to be unsuitable for sterol extaction [15]. In a comparison of seven extraction procedures developed for total lipids, fatty acids, cholesterol and other sterols from food products, Hubbard *et al.* [15] reported that the Folch method [16] [chloroform–methanol (2:1, v/v)] gave slightly higher sterol values than did the method of Bligh and Dyer [17]. The Folch method also gave significantly higher recoveries for cholesterol and other sterols compared with the hydrochloric acid–diethyl ether extraction system. Some workers have compared the Folch method or a modification of it [18] with other solvent systems for cholesterol extraction {*e.g.*, light petroleum–

TABLE I

LIPID EXTRACTION METHODS

Sample type	Extraction method	Lipid class	Comments	Ref.
Food	(1) 4 *M* HCl digestion and diethyl ether extraction (2) Folch (3) 6 others tested, unsatisfactory	Fatty acids	4 *M* HCl was chosen owing to the speed, simplicity and recovery	115
Food products	(1) 4 *M* HCl (2) Folch (3) Bligh and Dyer	Cholesterol and other sterols	Folch was recommended based on superior extraction of cholesterol	15
Egg yolk	(1) Light petroleum–ethanol (2) Folch	Cholesterol esters and cholesterol	No difference	19
Food	(1) Chloroform–methanol (2) Hot benzene (3) Acetone (4) Diethyl ether	Total lipids and cholesterol	Chloroform–methanol gave the highest yield for lipid and for cholesterol content	21
Ready-to-serve food	(1) Light petroleum using Soxhlet apparatus (2) Folch	Total lipids and cholesterol	Folch was better	20
Raw and cooked beef muscle	(1) Diethyl ether (2) Folch	Total fat	Folch gave 6.2% higher recovery for raw and 13.4% higher for cooked	116

ethanol (2:1, v/v) for egg yolk [19] or Soxhlet extraction with light petroleum for "ready to serve" food [20]} and observed no difference in cholesterol values. Kaneda *et al.* [21] compared four different solvent systems (Folch method, hot benzene, acetone and diethyl ether) for extracting lipids and cholesterol from food and found the Folch method to give higher percentage yields for both lipid and cholesterol from short-necked clam and top shell fish. Two popular methods for sample preparation include the official AOAC method [22] for multicomponent foods (based on the method of Punwar [13] which includes an initial extraction with chloroform–methanol–water [17]) and the method of Sheppard *et al.* [14] (based on the Folch method). Both of these methods involve multiple steps requiring homogenization of the sample with large volumes of solvent and quantitative transfer and filtering into a separating funnel. The extract is washed repeatedly with water or salt solution and centrifuged to separate the chloroform and aqueous phases. The chloroform phase is then passed through sodium sulphate and finally evaporated under nitrogen. In an attempt to simplify this lengthy procedure, Washburn and Nix [18] reported no difference in the amount of cholesterol extracted with or without the use of sodium sulphate or whether water or 0.88% KCl solution was used. Currently, the most suitable and commonly used method for the extraction of lipids and cholesterol from food is the Folch method (including all its modifications). For total lipid extraction, our laboratory uses and recommends a modified Folch method [23] that reduces both the solvent requirements and the number of wash steps.

Autoxidation of lipids can occur easily if they are exposed to air, light and heat, and precautions should be taken during sample preparation to ensure that no degradation or other alteration occurs. Cholesterol, having a double bond, can also undergo autoxidation in the presence of oxygen. To avoid or minimize autoxidation, samples should be stored at low temperature (−20°C or lower) and analysed as soon as possible to prevent enzymatic degradation before solven extraction. An excellent review of sample storage and handling before extraction of lipids has been published [24]. After extraction, samples should be kept in inert solvents under nitrogen in air tight vials and refrigerated to help reduce autoxidation. If fatty acids are of interest, an antioxidant such as 2,6-di-*tert.*-butyl-*p*-cresol (BHT) or pyrogallol is often added to the

extraction solvents to prevent oxidative degradation of unsaturated lipids. Many foods contain β-carotene and tocopherols, which are natural antioxidants that offer some protection to lipid extracts.

Maerker and Unruh [25] studied the autoxidation of cholesterol during saponification by two different methods, a dry column procedure [26] and the official AOAC procedure [22], and compared them with a sample without prior saponification. They reported that regardless of the method of saponification, it contributes to the generation of oxidation products and that even without saponification some oxidation products were present. They also found 0.16 μg of oxides formed as artifacts per milligram of cholesterol during their HPLC–GC procedure. Addition of 0.3% of BHT as an antioxidant in the dry column saponification of cholesterol did not result in reduction of oxidation products. It was concluded that autoxidation of cholesterol during analysis can be minimized but it cannot be completely eliminated. However, the amount of autoxidation products relative to the amount of cholesterol is very small and should not be significant in the determination of total cholesterol. Oles *et al.* [27] investigated factors affecting the recovery of cholesterol from baked food products and found that pyrogallol addition did not significantly alter the recovery.

2.2. *Direct saponification*

Recently there has been a trend towards simplifying sample preparation in order to reduce the solvent volume and shorten the analysis time. After extraction of lipids the usual next step is basic hydrolysis (saponification) to separate cholesterol and other unsaponifiable matter from fatty acids, which usually account for most of the extracted lipids. When cholesterol and other sterols are of primary interest, many workers have used direct saponification of the sample in order to eliminate the initial extraction step and thus simplify the procedure. Various workers have compared direct saponification for cholesterol determination to lipid extraction prior to saponification [23,28–33]. Kovacs *et al.* [31] found the cholesterol content in cod muscle determined using direct saponification to be higher than that determined by the AOCS official method [34]. Van Elswyk *et al.* [32] also obtained higher values for egg yolk cholesterol using direct saponification than with the AOAC official method [35] and concluded that direct saponification is more accurate. They obtained a value of 19.1 ± 0.4 [standard error (S.E.)] mg cholesterol per gram of yolk in the National Institute of Standards and Technology (NIST) reference material [cholesterol in whole egg powder which had a certified concentration of 19.0 ± 0.2 (S.E.)]. Others [30] have reported comparable or slightly higher cholesterol values in twenty different meat products using direct saponification and a 99.8% recovery of added cholesterol. Al-Hasani *et al.* [28], using a direct saponification method for cholesterol in frozen foods, found a superior recovery of cholesterol from spiked samples, 100.2% *versus* 94.9% for the AOAC method, and a good correlation ($r = 0.9996$) with the AOAC method. In our laboratory we have studied factors affecting the determination of cholesterol in egg yolk [23] and found no significant difference between using direct saponification or chloroform–methanol extraction prior to saponification. Other workers have also reported no difference between the two methods when analysing for total cholesterol in milk [33], egg products [29] and blood plasma [36].

The inconsistency in reported results could be due, in part, to the lack of standardization of conditions used for the saponification–extraction steps (Table 2). Factors contributing to variations in cholesterol determinations include sample size and composition, volume and strength of base, type of alcohol used, temperature and time of hydrolysis, the use of refluxing apparatus or capped tubes, type and volume of extraction solvent and post-extraction manipulations.

For precise results to be obtained for cholesterol determination, certain requirements must be met in the saponification and extraction steps. Cholesterol must be released from lipoprotein complexes, cholesterol esters must be completely hydrolysed and fatty acids held as soaps in the aqueous phase and thus separated during extraction. The extraction solvents should recover cholesterol quantitatively from the aqueous phase without forming emulsions. After a comprehensive review of the methodology for cholesterol determination in serum, De La Huerga and Sherrick [37] concluded that the saponification procedure of Abell *et al.* [38] was the most suitable. They suggested that the concentration of KOH in the final saponification solution should be between 0.33 and 0.5 *M* in ethanol and that choles-

TABLE 2

SAPONIFICATION AND EXTRACTION CONDITIONS FOR SAMPLE PREPARATION FOR CHOLESTEROL DETERMINATION

Sample matrix	Reagent[a]	Temperature (°C)	Time (min)	Extraction conditions	Ref.
0.5 g food (0.2 g oil)	10 ml EtOH + 2 ml KOH (50%, v/v)	70 (capped tube)	8	30 ml water + 20 ml hexane–diisopropyl ether (3:1)	86
Lipid extract	20 ml 0.5 *M* KOH in MeOH	Reflux	20	5 ml saturated NaCl + 10 ml *n*-heptane, repeat twice with light petroleum, wash 5 times with water	84
1 g oil	100 ml 0.8 *M* KOH in EtOH	80	30	Twice with 100 ml diethyl ether, wash with water, dry with Na_2SO_4	85
Lipid extract	2.5 ml 2% (w/v) KOH in EtOH	37	90	2.5 ml water + 5 ml light petroleum	8
Lipid extract	2 ml 1 *M* KOH in 93% EtOH	80	240	5 ml water + 3 times with 5 ml hexane	75
1 g egg noodles	1 ml 2 *M* KOH in MeOH	Reflux	30	60 ml 1.7% NaCl + twice with 100 ml diethyl ether–light petroleum (1:1)	45
0.5 g food	1 ml 50% KOH + 4 ml 95% EtOH	Reflux	60	2.5 ml water + 4 times with 5 ml hexane	31
1 g milk	1 ml KOH (80 g per 50 ml) + 4 ml EtOH	100	60	Solid-phase extraction	33
0.5 g egg product	10 ml 2.4 *M* KOH in EtOH	70	60	5 ml saturated NaCl + 5 ml water, 3 times with 10 ml light petroleum	29
<0.8 g lipid from meat	8 ml 50% KOH + 40 ml EtOH–MeOH (95:5)	Reflux	60	100 ml toluene, wash twice with 1 *M* KOH and 5 times with water, dry with Na_2SO_4	30
10 g frozen food	10 ml 60% KOH + 40 ml EtOH	Reflux	30	Twice with 50 ml toluene or hexane, wash 3 times with water, dry with Na_2SO_4	28
1 g egg	8 ml 50% KOH + 40 ml 95% EtOH	60 (capped tube)	60	40 ml water, 3 times with 60 ml hexane, wash 5 times with 100 ml water, dry with Na_2SO_4	32
0.2 g egg	0.6 ml 33% KOH + 9.4 ml 95% EtOH	60 (capped tube)	60	5 ml water + 10 ml hexane	23
1.5 g food	2 ml 50% KOH + 15 ml EtOH or iPrOH	(1) Ambient (2) Reflux	18 h 60	10 ml 10% NaCl, 7 ml toluene or hexane–diethyl ether (85:15), wash with 10 ml 10% NaCl, dry with Na_2SO_4	27
Lipid	1 ml 40% KOH + 20 ml EtOH	85	60	20 ml water, 3 times with 20 ml diethyl ether, dry with Na_2SO_4	21
Lipid	25 ml 1 *M* NaOH in EtOH	Reflux	60	3 times with diethyl ether, wash with water	20
5 g oil	50 ml 2*M* KOH in MeOH	Reflux	60	100 ml water, 5 times with diethyl ether, wash 3 times with water, dry with Na_2SO_4	40
0.2 g egg	6 ml 2.5% KOH in EtOH + 0.3 ml water	70	90	6 ml water, 12 ml light petroleum	19
Plant lipids	1 g KOH in 5 ml aqueous EtOH	Reflux	120	1 time with diethyl ether, wash with water, dry with Na_2SO_4	44
100 mg lipid	0.5 ml saturated KOH + 8 ml EtOH	80 (capped tube)	8	12 ml water, twice with 20 ml cyclohexane	42
Lipid	8 ml KOH (60 g/40 ml) + 40 ml EtOH–MeOH–iPrOH (90:5:5)	Reflux	60	100 ml benzene, wash once with 200 ml 1 *M* KOH, once with 40 ml 0.5 *M* KOH and 3 times with 40 ml water, dry with Na_2SO_4	22

[a] MeOH = Methanol; EtOH = ethanol; iPrOH = isopropanol (2-propanol).

terol esters are completely hydrolysed at 37–40°C in 60 min. In order to test the hydrolysis step of cholesterol esters to cholesterol in serum, Lillienberg and Svanborg [36] varied the hydrolysis time (from 5 to 120 min) at three different temperatures (35, 55 and 75°C). At 37°C they obtained higher values with

increasing hydrolysis time. Hydrolysis at 55°C gave significantly higher values up to 30 min, after which no further increase was observed. At 75°C slightly higher values were observed up to 15 and 30 min but the values were lower compared with hydrolysis at 55°C for 30 min. Both investigators used a hydrolysis solution consisting of 94 parts of 95% ethanol and 6 parts of 33% aqueous KOH [38]. This hydrolysis solution is routinely used in our laboratory for egg yolk cholesterol determination. Fresh egg yolk (0.2 g) and 10 ml of hydrolysis solution are heated at 60°C for 1 h in capped 50-ml tubes. The tubes are shaken occasionally during heating to bring into solution any sample adhering to the wall of the tube. It is important that the entire sample is evenly distributed in the hydrolysis solution. These conditions are sufficient for complete hydrolysis of cholesterol esters and also give clean samples without any free fatty acids as determined by GC analysis. In contrast, in our laboratory when studying phytosterols, a much larger sample size is used (2–5 g of dried egg yolk and 100 ml of hydrolysis solution) and free fatty acids consistently appear in the chromatograms in varying concentrations. To minimize contamination, all operations are performed in clean glass tubes with PTFE-lined screw-caps and all solvents are purified by distillation using an efficient fractionating column. If the samples contain very small amounts of cholesterol, contamination of the sample with flakes of skin might be a problem as skin contains relatively large amounts of cholesterol and other sterols [39].

More recently, Lognay *et al.* [40] studied the efficiency of the saponification extraction step for the extraction of sterols from edible oils using radiolabelled cholesterol and cholesteryl oleate. Using diethyl ether, one of the more commonly used solvents (Table 2), as extraction solvent after saponification, five extractions were required for the quantitative recovery of labelled cholesterol (96% recovery for spiked sunflower oil and 99% recovery for spiked butter oil). Significant differences were observed for the initial extraction recoveries from sunflower oil (60%) and butter oil (75%). Washing the ether phase three times with water accounted for a *ca.* 1% loss of cholesterol.

Oles *et al.* [27] studied some of the more significant factors affecting the recovery of cholesterol from various food matrices. Factors examined were: type of alcohol, extraction solvent, use of antioxidants, time and temperature of hydrolysis and spiking level. Hydrolysis conditions had a significant impact on the recovery of cholesterol when isopropanol was used as a solvent for hydrolysis but when ethanol was used there was no substantial effect. The extraction solvents compared were hexane–diethyl ether (85:15) and toluene, with the latter giving significantly higher recoveries.

In our laboratory, hexane was found to be an efficient solvent for the extraction of cholesterol from the saponification mixture of egg yolk. Standard additions from 1.2 to 6 mg of cholesterol yielded a recovery in excess of 98% with a single extraction for egg yolk samples of 0.2 g or less [23]. Similar results were obtained in experiments with [4-^{14}C]cholesterol [41], which showed that a single hexane extraction of the hydrolysis mixture extracted about 99% of the labelled cholesterol. Hexane is an excellent solvent because it is less toxic than other solvents commonly used and does not form emulsions as does toluene [30]. Being non-polar, it is more suited for the efficient partitioning of unsaponifiable matter into the organic phase and eliminates the necessity to dry the solvent with anhydrous sodium sulphate. It also does not form peroxides as does diethyl ether, which could cause the production of degradation products representing as much as 5% of the total sterols [40]. In our laboratory it was also observed that the amount of fat in the sample can affect the extraction of cholesterol. By increasing the amount of soybean oil added to a cholesterol standard (for 0 to 170 mg) the efficiency of the first extraction, as percentage of the total, decreased from 98.2% to 94.1% [23]. Slover *et al.* [42] also demonstrated that the presence of fat in the saponification mixture affected the extraction of both tocopherols and sterols and that two extractions with cyclohexane were needed for quantitative recovery.

Some workers found it necessary to purify the solvent extract further by either thin-layer chromatography (TLC) [43,44] or column chromatography [27,45]. Tsui [33] isolated cholesterol and an internal standard after saponification by solid-phase extraction on a non-polar adsorbent C_{18} cartridge instead of using a solvent extraction procedure. He described many critical steps in which losses of cholesterol can occur. It was emphasized that the adsor-

bent must be properly conditioned with methanol and then water and not be allowed to dry prior to application of the sample. The transfer of the rinse on to the adsorbent material should be quantitative, overloading of the cartridge should be avoided and the pH must be adjusted to between 2 and 5.

When diethyl ether alone or in combination with other solvents is used as an extraction solvent, some workers found impurities in the unsaponifiable matter [46]. These were primarily residual soaps [40] and extracted lipids (most likely free fatty acids) and they caused rapid contamination of the capillary column [27].

3. CHROMATOGRAPHIC ANALYSIS

3.1. Gas chromatography

3.1.1. Separation by packed versus capillary columns

Sterols of plant and marine origin are found in the unsaponifiable fraction of lipid extracts from foods and their presence may interfere with analysis for cholesterol by both spectrophotometric and chromatographic methods.

Plant sterols are biologically important cholesterol analogues which differ structurally in the presence of a methyl or ethyl group at the C-24 position or unsaturation of the side-chain. Although campesterol, β-sitosterol and stigmasterol are plant sterols which make up the major portion of the total sterol fraction of edible plant oils [2], significant amounts of other sterols can be present in various oils (*e.g.*, Δ^7-stigmasterol, brassicasterol and avenasterol).

Tocopherol, tocotrienol and their α, β, γ and δ isomers, which are widely distributed in foods of plant and animal origin, are also present in the unsaponifiable fraction. In foods of animal origin the α-tocopherol content is in the range 1–30 μg/g. The α-tocopherol content of plant material (grain, fruits and vegetables) ranges between 1 and 25 μg/g, with higher values for almonds and filberts, 270 and 210 μg/g, respectively [47]. In vegetables and seed oils other isomers occur in substantial amounts with the total tocopherol content in the range 40–2600 μg/g. In addition to sterols and tocopherols, other compounds found in the unsaponifiable matter include saturated hydrocarbons, squalene, aliphatic alcohols, terpene alcohols, triterpene alcohols and steryl esters [48–51].

Analysis for free and esterified sterols and triterpene alcohols (4-monomethyl- and 4,4-dimethylsterols) may be applied to characterize edible oils and to detect possible adulteration [50–54]. For example, the presence of margarine in butter can be detected by analysing for β-sitosterol, which is present only in vegetable oil. Similarly, substitution of cheaper rapeseed oil for olive oil would result in easily detectable brassicasterol, not found in olive oil, and substitution of animal-derived fat would result in an increase in cholesterol content.

The determination of sterols by GC on packed columns has been reviewed previously [55–57]. Retention characteristics for 50 free sterols and their trimethylsilyl (TMS) derivatives relative to cholesterol on several different stationary phases ranging in polarity from non-polar SE-30 to polar Silar 5-CP have been reported [55]. Retention times for 92 sterols and closely related compounds as steryl acetates were determined on four common stationary phases on packed columns [58]. More recently, Xu *et al.* [59] studied the chromatographic properties of TMS ethers of 100 sterols and related compounds and determined their retention times relative to cholesterol on an SE-30 packed column. Relative retention times of 168 acetate derivatives of sterols and triterpene alcohols have also been determined on OV-1 and OV-17 support-coated open-tubular (SCOT) glass capillary columns [60].

Although information obtained using packed columns was useful, packed columns have been largely replaced by wall-coated open-tubular (WCOT) fused-silica column technology in most laboratories during the past decade. Packed column instruments can easily be converted for use with 0.53 mm I.D. capillary columns (referred to as "megabore" or "halfmil") with currently available injector and detector adapters. These fused-silica columns offer a number of advantages over packed columns. WCOT columns which are surface bonded and cross-linked offer increased thermal stability and higher operating temperatures with significantly less bleeding. A lower level of column bleeding minimizes detector contamination, extends the lifetime of the column and makes reproducible peak integration easier. The high number of theoretical plates with capillary columns allows faster analysis times and improved resolution. The manufacturer's quality control testing on individual columns en-

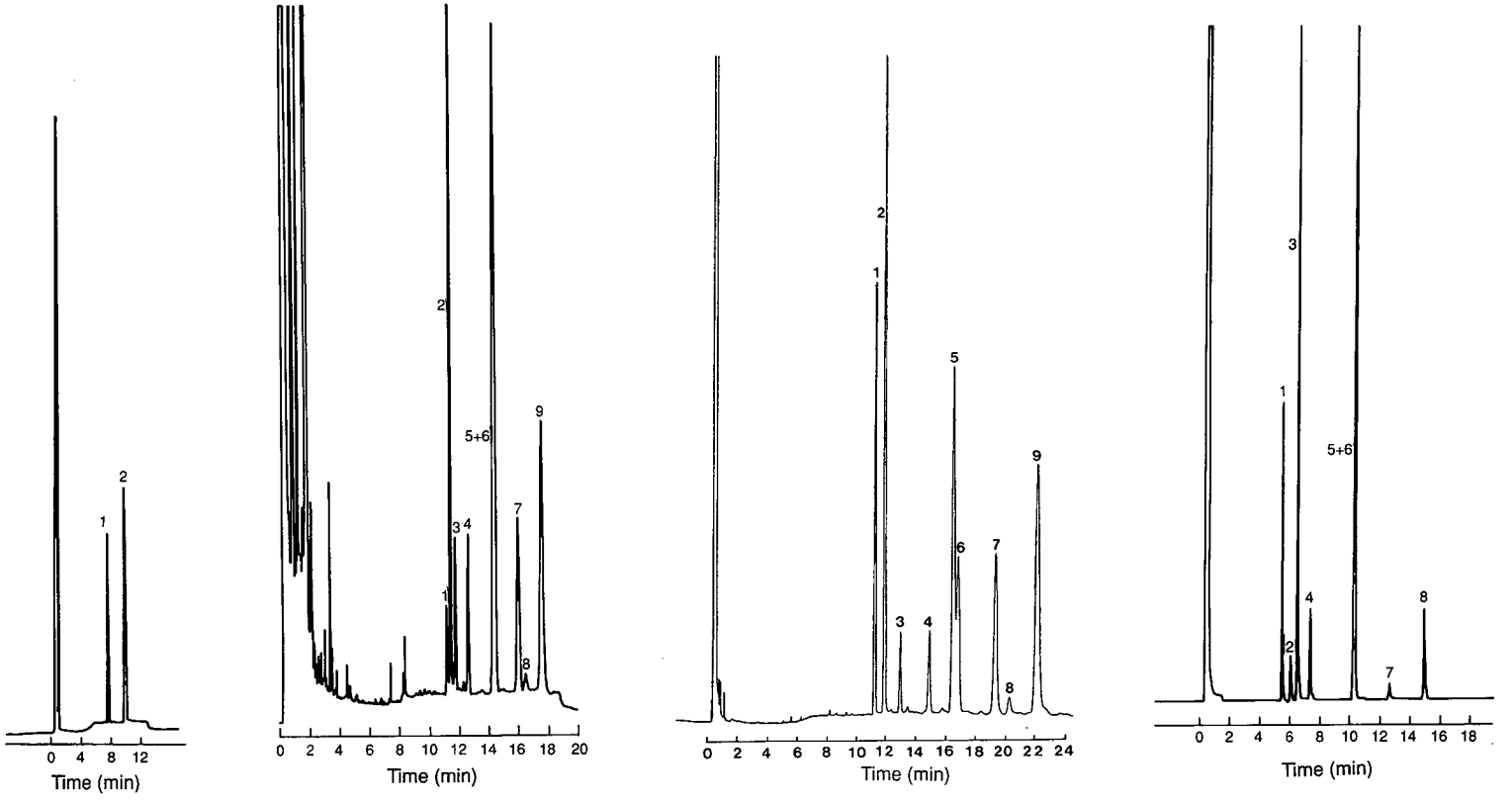

Fig. 1. Gas chromatogram of an egg yolk sample. Peaks: 1 = 5α-cholestane; 2 = cholesterol. Column, DB-5 (J&W Scientific, Folsom, CA, USA), 15 m × 0.53 mm I.D., 1.5 μm d_f; temperature, 70°C, then increased at 30°C/min to 270°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 270°C; carrier gas, helium at 70 cm/s.

Fig. 2. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 + 6 = α-tocopherol and cholesterol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Conditions as in Fig. 1.

Fig. 3. Gas chromatogram of underivatized standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = cholesterol; 6 = α-tocopherol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Column, SPB-5 (Supelco, Bellefonte, PA, USA), 15 m × 0.53 mm I.D., 1.5 μm d_f. Other conditions as in Fig. 1.

Fig. 4. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = δ-tocopherol; 3 = 5α-cholestane; 4 = γ-tocopherol; 5 + 6 = α-tocopherol + cholesterol; 7 = campesterol; 8 = sitosterol. Column, DB-17 (J&W Scientific) 30 m × 0.25 mm I.D., 0.15 μm d_f; temperature, 250°C, then increased at 5°C/min to 270°C; injector temperature, 270°C; detector temperature, 270°C; carrier gas, helium at 30 cm/s; splitting ratio, 15:1.

sures higher column to column reproducibility. The greater inertness of fused-silica and more extensive deactivation allow acidic and basic compounds to be analysed on the same column, giving better peak shapes for active compounds. A narrower and more symmetrical peak shape improves quantification by improving the integration accuracy, leading to lower limits of detection.

Although thin-film columns are preferred for the determination of high-molecular-mass, high-boiling-point compounds (to minimize the bleeding from the column), we achieved exceptionally good results on a short, thick-film megabore column [DB-5, 15 m × 0.53 mm I.D. with film thickness (d_f) 1.5 μm] for the determination of cholesterol in egg yolk. Fig. 1 shows a typical chromatogram of a sample after more than 6000 injections on this column. As can be seen, the cholesterol peak is sharp, without tailing. It shows no adsorption, and this is confirmed by the fact that the response factors have remained constant over a long period of use. Thick films cover the active silanol groups on the surface of the fused-silica, providing high inertness and preventing tailing and adsorption of polar compounds when analysed in the underivatized form. Alternatively, a standard SE-30 capillary column (30 m × 0.25 mm I.D., 0.25 μm d_f) showed increased tailing of the cholesterol peak with column use and a corresponding increase in response factors.

The short, thick-film column gives adequate separation for cholesterol determination in samples where cholesterol makes up most (>98%) of the sterols and tocopherols present (*e.g.*, egg yolk). However, as the resolving power of a column is inversely dependent on film thickness and column diameter [61], longer columns with smaller inner diameter and thinner film thickness are needed for the more complex separations requiring maximum resolution. In analyses of multi-component foods, in which sterols and tocopherols of plant and animal origin may be present in significant amounts together with cholesterol, very efficient capillary columns are required to prevent co-elution of compounds. The separation of cholesterol and cholestanol, which differs from cholesterol only in the absence of a double bond, is difficult to achieve. Noda *et al.* [44], using gas chromatography–mass spectrometry (GC–MS), showed that plant surface sterols often contain cholestanol as a minor component that is inseparable from cholesterol on pakced columns (*e.g.*, SE-30, OV-1, OV-101) but separation was possible on an SE-52 capillary column. The resolution between these two sterols was found to be affected by the carrier gas pressure and injection splitting ratio [62] and their order of elution was reversed on a polar capillary column (SP-2330) [63]. Other closely related sterols found in seafood, desmosterol, 22-dehydrocholesterol, 24-methylenecholesterol and brassicasterol [2,3], have retention times which are very close to that of cholesterol. Sterols of different molecular size are easily separated on non-polar columns but pairs of sterols differing in degree of unsaturation are better separated on polar phases. Although highly efficient capillary columns can improve the separation of critical pairs of sterols, factors such as changes in film thickness, type of phase, temperature programming and derivatization all may have an effect on separation.

Another difficult separation is that of cholesterol and α-tocopherol, which co-elute on many GC systems. With packed columns their retention times are nearly identical [14]. Figs. 2–7 show separations of a mixture of standards of cholesterol, 5α-cholestane, squalene, phytosterols and α-, γ- and δ-tocopherol using various columns and conditions (author's laboratory, unpublished data). Figs. 8–11 show separations of actual food analysis (pea soup sample was from local cafeteria and the mayonnaise sample was purchased at the supermarket). There is little information in the literature on the determination of cholesterol in the presence of significant amounts of other sterols and tocopherols.

3.1.2. Derivatization

Derivatization of cholesterol and other sterols improves the peak shape and may contribute to reduced retention time and improve sensitivity. Although a variety of other derivatives including butyryl esters [14] and acetates [60] have been employed, the formation of trimethylsilyl (TMS) ethers is preferred in the GC of sterols. TMS ethers offer higher thermostability and lower polarity and exhibit less tailing due to lack of adsorption to polar sites on the column. Many different silylating reagents are available for the preparation of TMS derivatives of sterols (Table 3). To optimize the

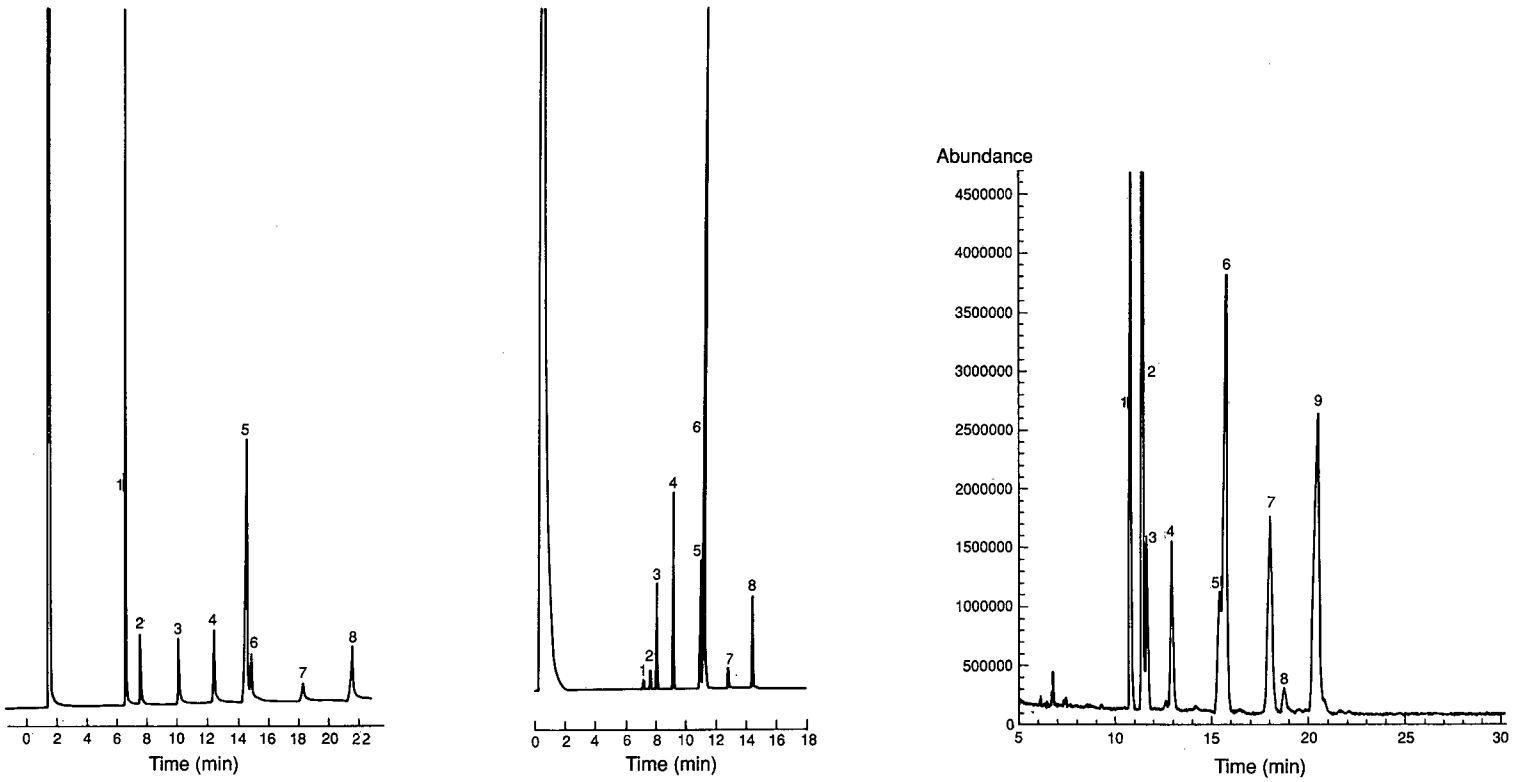

Fig. 5. Gas chromatogram of underivatized standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = sitosterol. Conditions as in Fig. 4.

Fig. 6. Gas chromatogram of TMS derivatives of standards. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = sitosterol. Column, RT_x-1 (Restex, Bellefonte, PA, USA), 30 m × 0.25 mm I.D., 0.25 μm d_f; temperature, 240°C, then increased at 5°C/min to 280°C; injector temperature, 270°C; detector temperature, 270°C; carier gas, helium at 25 cm/s; splitting ratio, 15:1.

Fig. 7. Separation of TMS derivatives of standards using GC–MS. Peaks: 1 = squalene; 2 = 5α-cholestane; 3 = δ-tocopherol; 4 = γ-tocopherol; 5 = α-tocopherol; 6 = cholesterol; 7 = campesterol; 8 = stigmasterol; 9 = sitosterol. Column, BP-5 (SGE International, Victoria, Australia), 25 m × 0.22 mm I.D., 0.25 μm d_f; temperature, 70°C, then increased at 30°C/min to 270°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 280°C; carrier gas, helium at 30 cm/s.

silylating conditions for cholesterol and its oxides, Nawar *et al.* [64] tested three silylating agents, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), BSTFA–1% trimethylchlorosilane (TMCS) and Sylon BTZ [N,O-bis(trimethylsilylacetamide (BSA): TMCS:trimethylsilylimidazole (TSIM) 3:2:3] and different time–temperature relationships. They found BSTFA–1% TMCS to give the best results within 1 h at 80°C. BSTFA is the preferred reagent because it produces hydrofluoric acid in the flame ionization detector, which reacts with the silicone to form the volatile SiF_4 preventing excessive build-up of silicon deposits in the detector. As cholesterol is poorly soluble in Sylon BTF [BSTFA–TMCS (99:1)] alone [39], pyridine was added during derivatization and then evaporated to eliminate the excessive tailing of the solvent peak during GC analysis. TMS reagents and derivatives are sensitive to moisture and therefore must be stored in tightly capped tubes and in moisture-free solvent to avoid hydrolysis. Also, the carrier gas must have an efficient moisture trap in-line to prevent hydrolysis of the TMS derivatives at high temperatures. Many silylating reagents are toxic, flammable and corrosive (chlorosilanes release hydrochloric acid on exposure to moisture) resulting in a need for proper ventilation in the sample preparation area and in the GC instrument area to remove vapours from the injector, detector and autosampler.

With improvements in thermal stability of liquid phases and with the development of inert fused-silica columns, many workers have found it unnecessary to prepare derivatives and prefer to determine sterols in the free form [21,23,29,57,65]. TMS derivatization not only adds an extra step in the procedure but could also contribute to increased noise, formation of artifacts, loss of sample and decreased linearity due to silicone deposits in the flame ionization detector [31,57]. Even on packed columns, Kovacs *et al.* [31] found that silylation did not result in a significant difference in sterol recovery. Van Delden *et al.* [20] reported good linearity and reproducibility for determination of cholesterol without prior derivatization as long as silylated supports were used. Kaneda *et al.* [21] obtained a good recovery and separation of cholesterol from other compounds from food without derivatization and, as the results were similar to those obtained using TMS and acetate derivatives, it was concluded that derivatization was not essential and that the unsaponifiable matter can be directly analysed by GC in order to save time. In our laboratory, we also found no significant difference for cholesterol content in egg yolk with or without derivatization (unpublished data). A mixture of cholesterol and eleven cholesterol oxide standards was analysed using on-column capillary GC with and without prior silylation [25]. The results showed that silylation changed the order of elution and that underivatized oxides were better separated from cholesterol than derivatized oxides on a 5% phenylsilicone column.

3.1.3. Quantitative analysis

3.1.3.1. Injection techniques. Split/splitless is the most commonly used injection technique for capillary GC but the recently available cool on-column injection systems have definite advantages. A more reproducible quantitative transfer of the sample to the column occurs than with split/splitless injection. Cold sample introduction ensures greater stability of thermally labile compounds and prevents discrimination and activity toward polar and high-boiling compounds as in the hot split/splitless injection. When using split/splitless injection, a significant reduction in peak degradation and tailing can be obtained through the use of silanized injection port inserts when analysing for polar compounds.

The cold on-column injection technique is most reliable for quantitative analysis [66,67] since the loss of sample due to thermal decomposition and irreversible adsorption on active sites in the column is minimized. With on-column injection, the sample is deposited directly into the highly inert fused-silica column at low temperature and then the injector temperature is raised rapidly to volatilize the sample. Normally a 5-m length of wide-bore (0.53 mm I.D.) deactivated fused-silica tubing, called a "retention gap" [68], is connected to the narrow-bore column with a glass butt connector. It traps nonvolatile residues and prevents damage to the liquid phase if dirty samples are injected. It also has the function of refocusing the sample, resulting in increased resolution and decreased peak splitting [66]. An alternative to on-column injection is "direct injection", in which the sample is deposited in a low-volume deactivated glass liner in the injection port. With these injection techniques the problems

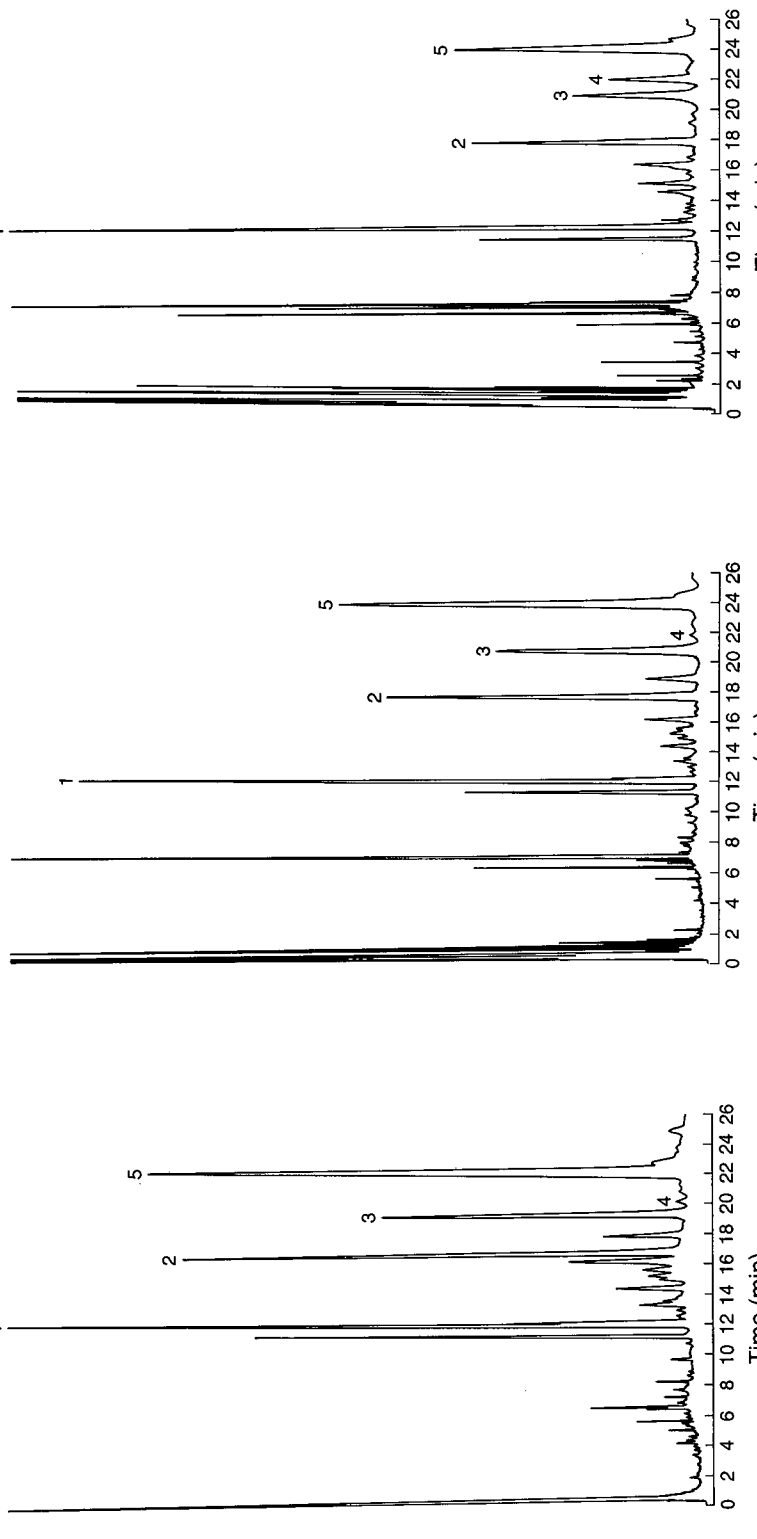

Fig. 8. Gas chromatogram of underivatized sample of split pea soup. Peaks: 1 = 5α-cholestane; 2 = cholesterol; 3 = campesterol; 4 = stigmasterol; 5 = sitosterol. Column, SPB-5 (Supelco, Bellefonte, PA, USA), 15 m × 0.53 mm I.D., 1.5 μm d_f; temperature, 70°C, then increased at 30°C/min to 270°C; on-column injection at 80°C, then increased at 150°C/min to 270°C; detector temperature, 270°C; carrier gas, helium at 70 cm/s.

Fig. 9. Gas chromatogram of TMS derivatives of split pea soup sample. Peaks and conditions as in Fig. 8.

Fig. 10. Gas chromatogram of TMS derivatives of mayonnaise sample. Peaks and conditions as in Fig. 8.

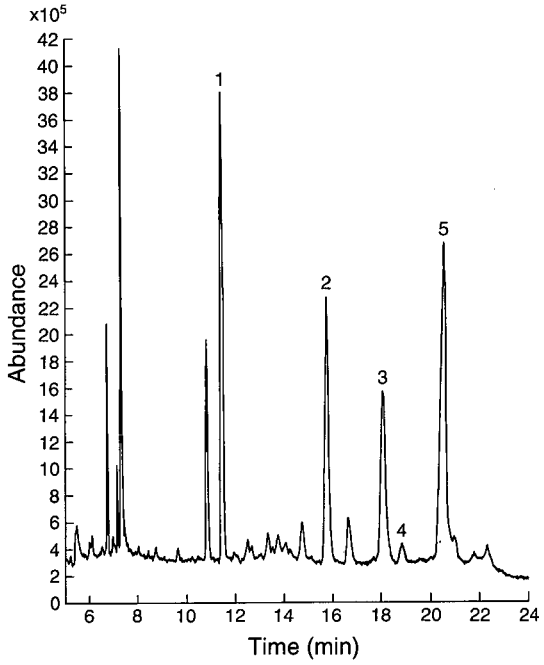

Fig. 11. Separation of TMS derivatives of split pea soup sample with GC-MS. Peaks: 1 = 5α-cholestane; 2 = cholesterol; 3 = campesterol; 4 = stigmasterol; 5 = sitosterol. Conditions as in Fig. 7.

caused by non-volatile sample residues deposited during injection can be easily overcome by either cutting off a section of the retention gap or cleaning the glass insert.

3.1.3.2. Detection and quantification. The most frequently used detection technique for cholesterol and other sterols is flame ionization detection (FID). It has good sensitivity and a wide linear range. Using a cooled on-column injector and an efficient capillary column (5% phenylsilicone), the detection limit for a cholesterol standard was determined to be 325 pg [25]. When helium is used as the carrier gas with capillary columns, nitrogen may be supplied as the make-up gas to improve the sensitivity of FID.

The low flow-rate of the carrier gas from the capillary column is an advantage in GC–MS in which the mass-selective detector is directly coupled to the GC column and requires a very low flow through the system. In the last decade, mass spectrometry coupled with GC or high-performance liquid chromatography (HPLC) has become an important and powerful technique for the accurate analysis of extremely complex mixtures in which compounds of unknown nature may co-elute with cholesterol. It provides high resolution along with positive identification of the individual components. Mass-selective detectors can yield sensitive and highly specific data and in combination with isotope-labelled internal standards provide highly accurate and precise measurements. Retention times in GC provide only tentative identification and the proof of structure is usually based on characteristic fragmentation patterns provided by MS. Isotope dilution mass spectrometry (IDMS) has been recognized as a reliable and highly accurate method for measuring cholesterol in serum [41,62,69,70] and in food matrices [71].

In isotope dilution (ID) analysis, isotopically labelled cholesterol ([3,4-^{13}C]cholesterol or cholesterol-d_7) is added to the sample to serve as an internal standard and after saponification, extraction and formation of TMS ethers, the ion intensity ratio of the molecular ions are measured by GC–MS. Gambert *et al.* [69] evaluated the accuracy of the GC method in comparison with isotope dilution mass fragmentation (IDMF) in the chemical ionization mode and found the correlation coefficient between the two methods to be 0.997. Takatsu and Nishi [70] determined total cholesterol in serum by electron impact IDMS using HPLC to separate cholesterol. When they compared the results with the conventional GC–IDMS method [41], the mean values obtained by the two methods agreed within 1%. The suitability of MS detection for HPLC has recently been reviewed [72]. High precision (coefficient of variation $<0.5\%$) can be achieved by GC–IDMS if close attention is paid to possible interferences and sources of analytical error [41,62].

The choice of internal standard and the step in the analytical procedure at which it is added to the sample are important for the determination of cholesterol. The internal standard should be added to the sample at the earliest possible step to compensate for losses which occur during extraction, transfers, filtration, evaporations and derivatization. A frequently used internal standard for cholesterol analysis is 5α-cholestane, a non-polar, saturated compound of similar chemical stucture which is easily extracted with non-polar solvents. As it does not have a hydroxyl group it elutes as a sharp peak well before cholesterol in GC. As in other GC analyses, to achieve accurate quantification, experi-

TABLE 3

GC CONDITIONS AND DERIVATIZATION PROCEDURES FOR CHOLESTEROL AND STEROL DETERMINATIONS

Sample type	Derivative[a]	Internal standard	Column	Temperature (°C)	Ref.
Cholesterol					
Eggs	(1) TMS (2) None	5α-Cholestane	(1) Packed OV-17 (2) Packed SP-2340	(1) 250 (2) 160–180	19
Serum	TMS (BSA)	[3,4-^{13}C]Cholesterol	25 m × 0.31 mm I.D., 0.25 μm d_f Ultra-1	295	62
Mixed diets	TMS	Cholestanol	25 m × 0.2 mm I.D., 0.11 μm d_f 5% phenyl methyl silicone	260	9
Food	TMS (DMF + BSTFA)	External standard	15 m × 0.32 mm I.D., 1.0 μm d_f DB-5	260	27
Food	None	Cholesteryl *n*-butyrate	Packed 5% SE-52	240	20
Food	TMS	External standard	Packed 0.5% Apiezon L		13
Food	None	5α-Cholestane	Packed 5% SE-30	260	21
Milk	TMS (BSTFA + TMCS)	5α-Cholestane	30 m × 0.25 mm I.D., 0.25 μm d_f DB-1	245–285	33
Meat	TMS (HMDS + TMCS)	5α-Cholestane	25 m × 0.32 mm I.D., DB-5	190–260	30
Food	TMS (HMDS + TMCS)	5α-Cholestane	Packed 0.5% Apiezon L	235	28
Eggs	TMS (HMDS + TMCS)	5α-Cholestane	Packed 3% SP-2401	230	32
Egg products	None	5α-Cholestane	15 m × 0.25 mm I.D., 0.1 μm d_f DB-52	275	29
Egg yolk	None	5α-Cholestane	30 m × 0.25 mm I.D., 25 μm d_f SE-30	70–300	23
Coconut oil	TMS (BSA)	Isotopically labelled cholesterol	Packed 1.5% OV-101	230	71
Serum	TMS (BSA)	[3,4-^{13}C]Cholesterol	50 m × 0.3 mm I.D., SE-54	280	70
Serum	TMS (BSA)	Cholesterol-d_7	Packed 1.5% OV-101	250–265	41
Serum	TMS (BSTFA + TMCS)	(1) Epicoprostanol (2) 5α-Cholestane (3) [3,4-^{13}C]Cholesterol	25 m × 0.22 mm I.D., SE-30	250–285	69
Sterols					
Food	Butyryl esters	5α-Cholestane C_{28} hydrocarbon C_{30} hydrocarbon C_{32} hydrocarbon	Packed 1% SE-30	250–260	14
Food	(1) None (2) TMS	5α-Cholestane	Packed 3% OV-17	230	31
Fats and oils	TMS (BSTFA + TMCS)	5,7-Dimethyltocol	50 m × 0.25 mm I.D. Dexsil 400	260	42
Soybean hulls	TMS (Sylon BTZ)	5α-Cholestane	12 m × 0.25 mm I.D., 0.1 μm d_f DB-1	100–265	117
Serum and dietary supplements	None	5α-Cholestane	10 m × 0.26 mm I.D., CP-Sil 8	220–245	65
Serum	TMS (BSTFA)	Epicoprostanol	25 m × 0.3 mm I.D., OV-1	240	74

[a] TMS = Trimethylsilyl; BSA = N,O-bis(trimethylsilyl)acetamide; BSTFA = N,O-bis(trimethylsilyl)trifluoracetamide; DMF = N,N-dimethylformamide; HMDS = hexamethyldisilazane; TMCS = trimethylchlorosilane.

mentally derived response factors are used to compensate for the varying response of the flame ionization detector to different compounds and losses resulting from other manipulations. Reference standards of a known amount of internal standard and cholesterol, preferably a minimum of three varying concentrations bracketing the sample, should be carried through the entire procedure to determine the relative response factor for quantification and to establish linearity of response. By analysing the reference standards regularly the entire analytical process can be monitored to evaluate the errors introduced by differences in reagents and variations in GC performance resulting from irreversible adsorption on the column, accumulation of contaminants in the injector and leaks. Use of procedures which omit internal standardization should be strongly discouraged as there is no common control over each individual sample throughout the procedure. Other compounds suggested as internal standards include 5,7-dimethyltocol [43], cholesteryl *n*-butyrate [20], cholestanol [9] and betulin [43,75]. Epicoprostanol (a sterol differing from cholesterol only in the lack of a double bond) has been used as an internal standard in the GC determination of cholesterol in serum [69,74]. Its suitability as an internal standard was tested [74] by determining relative recovery of epicoprostanol and cholesterol after saponification, extraction and derivatization steps and was found to be not significantly different from that obtained by direct derivatization of a cholesterol–epicoprostanol mixture.

3.2. Liquid chromatography

Although HPLC has become a very useful analytical tool in lipid analysis in general [24,75] and can offer a non-destructive alternative to GC techniques, its specific application to cholesterol determination has been limited because cholesterol does not have a strong absorption peak in the UV region. Cholesterol and related sterols do, however, have an unsaturation centre and a functional group that absorbs in the range 203–214 nm [46] with a maximum at 205 nm for cholesterol [59]. HPLC offers the advantage that many separations can be achieved at ambient temperature and the separated compounds can be recovered from the mobile phase for further analysis by complementary techniques such as GC [51] and MS [70].

Because cholesterol and cholesterol esters may play an important role in the diagnosis of certain diseases, their concentrations need to be determined separately. Many HPLC methods have been published for the determination of free cholesterol, individual cholesterol esters and cholesterol metabolites [76,77] in plasma [78,79] and other biological samples [80,81]. Normal-phase columns have been used to separate triglycerides, diglycerides, sterols, free fatty acids and monoglycerides after removal of phospholipids by column chromatography but reversed-phase separations have generally been preferred in the determination of cholesterol. Vercaemst *et al.* [82] achieved good HPLC separations of free cholesterol and cholesterol esters from both macrophage cells and human low-density lipoproteins by isocratic elution with acetonitrile–isopropanol (50:50, v/v) on a reversed-phase column (Zorbax ODS) with detection at 210 nm. Lipids were extracted with hexane–isopropanol (3:2, v/v) and cholesteryl heptadecanoate was used as an internal standard. Similar separations were obtained [83] using a linear gradient of water (3 to 0%) in acetonitrile–tetrahydrofuran (65:35, v/v) and UV detection at 213 nm. Earlier reports on the application of reversed-phase HPLC to the determination of free and total cholesterol in serum [79] after isopropanol extraction of lipids resulted in poor separation of cholesterol and cholesterol esters when triglycerides were present. Newkirk and Sheppard [84] also observed that triglycerides, which comprise most of the total lipids in food, interfere with cholesterol detection and quantification and recommended the removal of the fatty acids from initial lipid extracts by saponification as a critical step in the HPLC determination of cholesterol.

To improve the measurement of total cholesterol in foodstuffs, Newkirk and Sheppard [84] converted cholesterol to its benzoate ester by reaction of the non-saponifiable fraction of the lipid extract with benzoyl chloride in pyridine. Amounts as low as 10 ng of cholesteryl benzoate were detected when using a reversed-phase column (μBondapak ODS) with a methanol mobile phase and UV detection at 230 nm. A variety of foods were analysed and the levels determined compared favourably with those obtained by GC [14]. Goh *et al.* [80] reported a sensitive method for desmosterol, 7-dehydrocholesterol and cholesterol in biological material by oxida-

tive conversion of the sterols with cholesterol oxidase into their conjugated enone forms followed by HPLC determination. Even higher sensitivities (2 pg for cholesterol and 3 pg for cholestanol) were obtained for cholesterol and cholestanol in plasma by converting them into the corresponding fluorescent carbamate esters prior to analysis. Following derivatization with 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide in benzene, the derivatives were separated on a reversed-phase C_8 column with acetonitrile–methanol–water (81:9:10, v/v/v) as eluent and detected spectrofluorimetrically with excitation and emission wavelengths of 360 nm and 440 nm, respectively [78].

The total cholesterol content has also been determined in the non-saponifiable lipid fraction of milk [46] and egg noodles [45] by HPLC. After clean-up by use of Sep-Pak silica gel to remove interfering substances, HPLC of total cholesterol was completed using a reversed-phase column (Alltech ODS), a hexane–isopropanol (99.9:0.1, v/v) mobile phase and detection at 205 nm. Beyer and Jensen [8] used HPLC to separate cholesterol from other compounds in the unsaponifiable matter in egg yolk and showed that 17.5% of the total cholesterol content as determined by the spectrophotometric method of Zlatkis *et al.* [5] was due to the presence of non-cholesterol compounds.

Foods may contain a variety of ingredients including plant and vegetable oils that contain phytosterols and tocopherols. The presence of these compounds may interfere with the quantitative separation of cholesterol. Holen [85] investigated the influence of various mobile phases and different column temperatures with reversed-phase C_8 and C_{18} columns on the separation of eight structurally closely related sterols, desmosterol, ergosterol, brassicasterol, fucosterol, cholesterol, stigmasterol, campesterol and sitosterol. The optimum column temperature was found to be 30°C and elution with methanol–water (99:1, v/v) gave a superior separation *versus* acetonitrile–water (100:0 to 95:5). Although the C_{18} column was found to be superior to the C_8 column for separation of cholesterol from most other sterols, the separation of cholesterol and fucosterol was poor and sensitivity was low (0.4 μg of cholesterol).

To detect cholesterol, phytosterols and tocopherols simultaneously in food, Indyk [86] used UV detection at 212 nm to monitor sterols and fluorescence detection (excitation at 295 nm, emission at 330 nm) in series to measure tocopherols. Using a reversed-phase C_{18} column (Rad-Pak) and a methanol mobile phase, cholesterol and the main phytosterols (stigmasterol, campesterol and sitosterol) were resolved (although not to the baseline) and well separated from isomers of tocopherol. With a hexane–isopropanol (99.9:0.1, v/v) mobile phase, phytosterols were eluted as a single peak before cholesterol. Although a satisfactory separation can be obtained at ambient temperature, as above, temperature and polarity of the solvent may be used to improve separations between structurally similar sterols [59]. For example, it was noted that cholesterol and lanosterol did not separate on a reversed-phase C_{18} column at 25°C but when the temperature was increased to 40°C partial separation was achieved.

Products of cholesterol oxidation have been found in food and, because they have been implicated in adverse human health effects [87,88] such as cytotoxicity, mutagenicity, carcinogenicity and angiotoxicity, many papers have been published on this subject in the last decade. Various amounts of cholesterol oxidation products have been found in cholesterol-containing food products that have been processed or stored under oxidizing conditions, including egg yolk powder [89–93], pork muscle [94], heated lard [95], tallow [96], butter, cookies and cakes [97] and infant formulas [98]. The level of oxidation products relative to the cholesterol concentration is very low. For example, in egg yolk powder the level of total cholesterol oxides ranged from undetectable to 311 ppm of total lipids [99] and from 3.6 to 6.2 ppm dry mass in mixed diets [100].

Many techniques have been used to separate and determine cholesterol oxidation products. They involve isolation and prefractionation steps on the extracted lipids using TLC [90,101], silicic acid column chromatography [64,100], semi-preparative HPLC [25,89,99] or saponification [90,95,97]. Quantification has been achieved by HPLC with normal-phase columns using UV detection and various mobile phases [91,92,94,101], capillary GC of TMS derivatives [64,93,97,100,102] or GC–MS [89,93,97, 100].

3.3. Other chromatographic methods

3.3.1. Complementary liquid column chromatographic procedures

Animal and plant tissues contain complex lipid mixtures of both polar and non-polar nature. The application of chromatographic techniques to the separation of lipids has been comprehensively reviewed [24,103–107]. Preliminary fractionation into polar and non-polar lipid classes is normally done by adsorption column chromatography. The total lipid extract is applied to a short column of silica gel or commercially available prepacked cartridges (*e.g.*, Sep-Pak or Bond Elut) and neutral lipids are eluted with chloroform and polar lipids with methanol [24]. Individual lipid classes can be eluted from these columns with increasing concentration of diethyl ether in hexane. Typically, cholesterol esters would be eluted with 2% diethyl ether in hexane followed by triglycerides with 5%, diglycerides and cholesterol with 15% and monoglycerides with 100% diethyl ether, and phospholipids can then be eluted with methanol [24].

Another approach to the separation of neutral and polar lipids is by the dry column method [26], in which simultaneous lipid extraction and separation of neutral and polar lipid classes are accomplished by sequential extractions from a dry column. In this procedure samples of meat or meat products are ground with anhydrous sodium sulphate and blended with Celite 545 and packed into a glass column on top of the trapping material, which is calcium hydrogenphosphate–Celite 545 (1:9). Neutral lipids are then extracted with dichloromethane followed by dichloromethane–methanol (9:1) to elute the polar lipid fraction.

3.3.2. Thin-layer chromatography

TLC is a relatively rapid and powerful method for the separation of individual lipid classes from a total lipid extract, but it is rarely used for quantitative purposes. Cholesterol is present in food as both free cholesterol and its esters and can be separated by TLC to allow individual determination by other chromatographic means. Most lipid class separations are carried out using silica gel G adsorbent layers and hexane–diethyl ether–formic acid (80:20:2, v/v/v) as the solvent system [106]. This results in the separation of cholesterol esters at the solvent front followed by triglycerides, free fatty acids, free cholesterol, diglycerides, monoglycerides and phospholipids at the origin. Bands are detected and eluted with chloroform–methanol (2:1, v/v) [103] for further quantitative analysis.

TLC or liquid column chromatography has also been used for sample clean-up after saponification and before GC [27,43] or HPLC [45,46,98] in the determination of cholesterol. Kaneda *et al.* [21], however, found that the procedures used to remove impurities (TLC, column chromatography and precipitation with digitonin) from the unsaponifiable fraction decreased the cholesterol recoveries and should be omitted. Tsui [33] used C_{18} Bond Elut cartridges to isolate cholesterol after saponification instead of solvent extraction.

Other applications of TLC in cholesterol determination include the analysis of intact steryl esters by HPLC after TLC of the Folch extract and the analysis of TMS ethers of cholesterol and cholesterol oxides by capillary GC after their separation on TLC plates from other unsaponifiable matter.

3.3.3. Thin-layer chromatography with flame ionization detection

A combination of TLC and FID in the Iatroscan analyser has provided a means for the determination of separated lipid components. This technique has found widespread application in a variety of fields and is particularly suited for the analysis of fats and oils. The TLC–FID system, developed in the early 1970s, has undergone many improvements, including new Chromarods, a new collector, an improved semi-automatic sample spotter and data acquisition [108]. These have resulted in improved quantification, linearity of response and sensitivity [109]. Although it has the advantages of high sample throughput, minimum sample preparation and can determine both free and esterified cholesterol, its application to the determination of cholesterol in various food matrices is limited owing to a lack of resolution between cholesterol and other sterols which are similar in molecular structure. In foods where cholesterol makes up $>98\%$ of total sterols or where only total sterols are of interest, TLC–FID was reported to give results that were not significantly different from those obtained by GC [110]. Lipids were extracted from sea food samples, saponified and the non-saponifiable matter extracted with

hexane. Although both internal and external standard methods gave linear calibration graphs for amounts of cholesterol in the range 1–20 μg applied to the Chromarods, the use of an internal standard was preferred for accurate determination because it minimized the effect of rod-to-rod variation. Comparison between TLC–FID and GC techniques for the determination of total cholesterol, fatty acids and plasmalogens gave very similar data but the reproducibility of the TLC–FID system was lower [111]. The inter-rod reproducibility was found to be too large, requiring determination of a response factor for each lipid component on individual Chromarods, and thus to improve the accuracy and precision each rod was considered as an individual analytical unit. Some workers [112] have reported similar results for the determination of lipid classes by TLC–FID and TLC followed by GC, but others [113] have found the accuracy and reproducibility of TLC–FID to be unacceptable. In applying TLC–FID to the determination of cholesterol in lipid extracts from chicken plasma, we found that the relative response factor varied with the concentration of cholesterol in the standard mixture, which created problems for accurate quantification. Reliable quantification can only be achieved if a suitable internal standard is used and peak areas are corrected using proper response factors. Response factors can be affected by several variables, including sample size, speed of scanning and flow-rate of hydrogen to the detector [114].

A variety of solvent systems can be used for the development of the Chromarods, depending on the specific application, but that most commonly used to separate total lipids into lipid classes is hexane–diethyl ether–acetic acid (or formic acid) (80:20:1, v/v/v). We have found that benzene–chloroform–acetic acid–methanol (50:10:0.3:0.2, v/v/v/v) is also a good solvent system for resolving these lipid classes. A good reference text on TLC–FID principles and applications has recently been published [114].

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Review

Liquid chromatographic purification and detection of anabolic compounds

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ABSTRACT

The role of liquid chromatography within methods of analysis for steroids, related compounds and β-agonists in biological samples is discussed. Special attention is given to the application of liquid chromatography in sample preparation and extract clean-up. Different forms of liquid chromatography, including immunoaffinity chromatography, are compared and evaluated. Methods for confirmation based on gas chromatography–mass spectrometry and cryotrapping Fourier transform infrared spectrometry are discussed.

CONTENTS

1. INTRODUCTION

Throughout the EEC, the use of xenobiotic anabolic agents is prohibited in food-producing animals. However, for over 30 years a variety of compounds have been used for purposes of growth promotion. The number of compounds used within a particular group, *e.g.*, steroid hormones, is still increasing and new classes of compounds find their way into the "black market' of growth promoters.

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One example of the latter is are the phenylethanolamines, primarily developed as human or veterinary drugs working on the β_2-adrenergic receptor. Well known examples of these so-called β-agonists are clenbuterol and salbutamol.

During the past 30 years, analytical chemists have developed a variety of analytical methods to monitor the presence of residues in biological samples. Liquid chromatography (LC) has been demonstrated to be a key technique. The relative importance is to a large extent based on the wide variety of separation mechanisms and application forms, ranging from binary solid-phase extraction (SPE) to highly efficient, small-particle high-performance liquid chromatographic (HPLC) systems. These different forms allow the use of LC during both sample preparation (clean-up) and final analysis.

The objectives of an analysis can be very different. Within residue analysis for (illegal) growth-promoting agents the compounds used are frequently exogenous. In these cases, as there are no tolerance levels, only unambiguous identification is necessary. However, there are instances where in addition to identification quantification is also necessary, *e.g.*, in forensic analyses for estradiol and testosterone, which are endogenous compounds also used for growth promotion. These different objectives of an analysis impose different demands on the techniques and procedures used. Attention will be focused, however, on methods suitable for combination with spectroscopic procedures for detection and identification.

Analytical methods for residue analysis as a rule contain three different steps: (i) preparation of a primary extract, (ii) extract clean-up and (iii) detection, identification and determination. Frequently, however, the distinction between these steps is not very clear. In a number of methods the detector is placed on-line with the equipment used for extract clean-up, *e.g.*, in HPLC with UV detection. However, also within these applications HPLC can be considered as an extract clean-up procedure prior to detection.

A detailed discussion of methods for the preparation of a primary extract is beyond the scope of this paper, even though it is often neglected. Especially the extraction of analytes from tissues is frequently not evaluated in sufficient detail, the main reason being the absence of good reference materials with incurred residues.

The use of HPLC and SPE techniques is part of most, if not all, modern analytical procedures for steroids and β-agonists at residue levels. Immunoaffinity chromatography (IAC) is one of the more recent additions to the number of techniques available to the analyst. IAC is not a new technique, but within residue analysis applications are relatively recent [1–7], of special interest being multi-residue applications. The use of IAC has been reviewed before [8]. IAC combines the ease of use of SPE with the selectivity of advanced chromatographic (HPLC) systems.

The number of detectors that are sensitive and selective enough to be applied on-line with LC is limited because the solvents used are not compatible, *e.g.*, immunochemical detection after reversed- or normal-phase LC, and the technology of coupling is still under development and not (yet) available in a large number of laboratories not specialized in this technique, *e.g.*, with LC–MS. Therefore, LC separations are frequently followed by off-line detection. Within toxicological and residue analysis, immunochemical and mass spectrometric techniques are widely used, the latter most frequently in combination with gas chromatography (GC–MS).

2. ANALYTICAL PROCEDURES

This section describes analytical procedures for the detection and identification of anabolic steroids and related compounds and of β-agonists in biological materials. The procedures are based on spectroscopic methods for identification.

2.1. Determination of anabolic steroids and related compounds

Methods used for the determination of anabolic compounds are frequently multi-residue methods based on SPE, IAC or HPLC for extract clean-up. Here the procedures suitable for extract clean-up are described and compared. Details can be found in the EEC manual on reference methods and materials [9].

The analytes involved are summarized in Table 1. Fig. 1 shows structures of representatives of the different groups of compounds, the androgens NT, T, MT, Bol and Tb, the estrogens EE2, E2, DES, DE, HEX and Z and the gestagens MPA, CMA and MGA.

TABLE 1

STEROIDS AND RELATED COMPOUNDS USED FOR GROWTH PROMOTION

Analyte	Abbreviation	CAS Registry No.	Formula	Molecular mass
17β-19-Nortestosterone	βNT	434-22-0	$C_{18}H_{26}O_2$	274.3
17α-19-Nortestosterone[a]	αNT	4409-34-1	$C_{18}H_{26}O_2$	274.3
17β-Testosterone	βT	58-22-0	$C_{19}H_{28}O_2$	228.4
17α-Testosterone[a]	αT	99-999-9	$C_{19}H_{28}O_2$	288.4
17α-Methyltestosterone	MT	58-18-4	$C_{20}H_{30}O_2$	302.4
Boldenone	BOL	846-48-0	$C_{19}H_{26}O_2$	286.4
17β-Trenbolone	βTb	10161-33-8	$C_{18}H_{22}O_2$	270.4
17α-Trenbolone[a]	αTb	80657-17-6	$C_{18}H_{22}O_2$	270.4
17α-Ethynylestradiol	αEE2	57-63-6	$C_{20}H_{24}O_2$	296.4
17β-Estradiol	βE2	50-28-2	$C_{18}H_{24}O_2$	272.2
Medroxyprogesterone acetate	MPA	71-58-9	$C_{23}H_{24}O_2$	386.5
Clormadinone acete	CMA	302-22-7	$C_{23}H_{24}ClO_4$	405.0
Megesterol acetate	MGA	595-33-5	$C_{24}H_{32}O_4$	384.5
Zeranol	Z	26538-44-3	$C_{18}H_{20}O_5$	322.4
Taleranol[a]	TAL	42422-68-4	$C_{18}H_{20}O_5$	322.4
Diethylstilbestrol	DES	56-53-1	$C_{18}H_{20}O_2$	268.4
Dienstrol	DE	84-17-3	$C_{18}H_{18}O_2$	266.4
Dexestrol	HEX	84-16-2	$C_{18}H_{22}O_2$	270.4

[a] Not an anabolic itself but a metabolite of the previous compound.

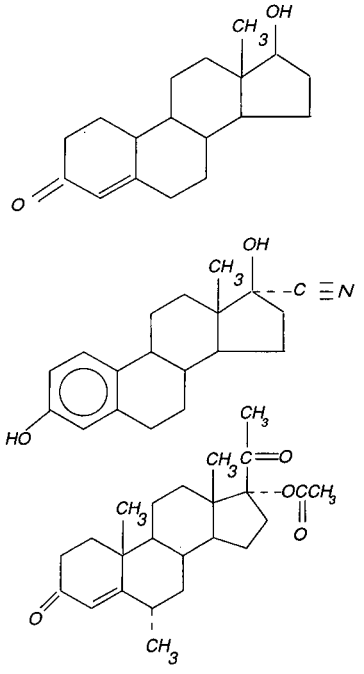

Fig. 1. Molecular structures of nortestosterone (top), ethynylestradiol (middle) and medroxyprogesterone acetate (bottom).

2.1.1. Sample preparation

If available [10], deuterated internal standards are added to the samples prior to analysis for accurate determination and control of false-negative results. Liquids samples (urine or bile) are incubated in the presence of 0.1 ml of β-glucuronidase–sulphatase (*e.g.*, suc'*Helix pomatia* containing 100 000 units of β-glucuronidase and 10 000 units of sulphatase) to deconjugate glucuronide and sulphate conjugates of the analytes (pH 5.2, 2 h at 37°C). After incubation the test portions are extracted with *tert.*-butyl methyl ether (TBME). The combined extracts are evaporated to dryness under a stream of nitrogen in a water-bath at 50°C.

Tissue samples can be extracted either by an enzymatic procedure with subtilisin A [11] or by mechanical (ultrasonic) extraction [9]. As anabolic steroids are frequently injected into the animal in the form of esters, an incubation under alkaline conditions can be applied (KOH in ethanol, 0.5 h at room temperature). After defatting, the extract is ready for further purification.

2.1.2. Extract clean-up with LC

2.1.2.1. Solid-phase extraction. SPE is frequently used for extract clean-up [12,13]. Both normal-

phase, based on silica-type materials, and reversed-phase methods, mainly with C_{18}-modified materials have been described. Typical procedures and elution behaviour are as follows (Sep-Pak columns, Waters–Millipore). With a silica cartridge, the cartridge is washed subsequently with 2 ml of ethanol and 5 ml of isooctane and the residue of the primary extract is dissolved in 5 ml of isooctane and applied to the cartridge. Analytes are eluted with ethanol–isooctane mixtures. With a C_{18} cartridge, the cartridge is washed with 2 ml of methanol and 5 ml of water and the residue is dissolved in water and applied to the cartridge. Analytes are eluted with methanol–water mixtures.

2.1.2.2. Immunoaffinity chromatography. For most of the compounds clean-up by IAC is possible. Several institutes have prepared their own materials. More important, however, several commercial firms supply suitable materials. For multi-residue methods appropriate IAC materials can be combined (MIAC). The procedure for sample preparation by IAC depends on the characteristics of the IAC material used [8]. The following procedure is suitable for the polyclonal rabbit antibodies tested. IAC materials usually have a capacity of 20 ng or more per ml of gel. For multi-residue analysis combinations of gels can be applied. The primary extract is dissolved in 0.05 ml of ethanol. Subsequently, 5–10 ml of water are added and the total mixture is applied to the IAC column. After sample application the column is washed with 5 ml of water and eluted with 5 ml of ethanol–water (1:1, v/v). The eluate is evaporated to dryness under a stream of nitrogen in a water-bath at 50°C or alternatively extracted with TBME. The dry residue is suitable for analysis by GC–MS. Fig. 2 shows the elution of nortestosterone from an IAC column as a function of the ethanol content of the eluent.

2.1.2.3. High-performance liquid chromatography. In addition, clean-up by reversed-phase LC is very effective. The following reversed-phase system has proved to be suitable for extract purification prior to GC–MS: precolumn, Chromguard reversed-phase cartridge; analytical column, Hypersil ODS C_{18} (150 mm × 7.5 mm I.D.); flow-rate, 1.5 ml/min.

The residue is dissolved in 0.10 ml of the HPLC eluent, of which subsequently 0.09 ml is injected into the system. The fractions of interest are collected, usually starting 0.5 min before the retention time of the analyte and ending 1 min later. Sometimes it is possible or advantageous to combine different analytes in a single fraction. In Table 2 an overview is given of suitable solvent compositions and retention times.

The eluent is removed under a stream of nitrogen in a water-bath at 50°C. In a number of instances on-line detection of the analytes at this stage is possible, *e.g.*, in the analysis of highly concentrated extracts of application sites or in samples of urine from veal calves. More details of these methods and the possibilities of, *e.g.*, identification using diode-array UV detection, are reviewed separately.

The choice between SPE, IAC and HPLC depends on a number of parameters: (i) availability; (ii) analyte(s); (iii) number of samples; and (iv) limit of detection needed. To illustrate the efficiency of the different techniques, a comparison was made of the quantitative determination of 17β- and 17α-nortestosterone (NT) in bovine urine. Three test portions of a single sample were pretreated as described. The extracts were purified on an SPE (C_{18} cartridge or an IAC or HPLC column. Fig. 3 shows the GC–MS ion chromatograms (as HFB derivatives) for the molecular ions of 17β- and 17α-NT (m/z = 666) and for the internal standard 17β-NT-d_3 (m/z = 669, added at a level of 2 μg/l).

From these chromatograms it can be concluded

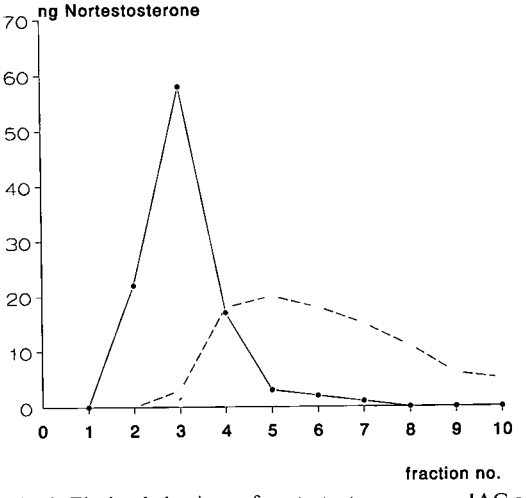

Fig. 2. Elution behaviour of nortestosterone on an IAC column. ● = Ethanol–water (50:50, v/v); dashed lined = ethanol–water (20:80, v/v).

TABLE 2

SOLVENT SYSTEMS AND APPROXIMATE RETENTION TIMES

Compound	Eluent	Retention time (min)
Diethylstilbestrol	CH_3OH–H_2O (65:35)	5.3
Dienestrol	CH_3OH–H_2O (65:35)	6.2
Hexestrol	CH_3OH–H2O (65:35)	5.7
17β-Estradiol	CH_3OH–H_2O (65:35)	4.5
Zeranol	CH_3OH–CH_3CN–H_2O (4:38:58)	4.8
Ethynylestradiol	CH_3OH–H_2O (65:35)	5.0
17β-Trenbolone	CH_3OH–CH_3CN–H_2O (4:38:58)	3.5
17α-Trenbolone	CH_3OH–CH_3CN–H_2O (4:38:58)	4.1
17β-Nortestosterone	CH_3OH–H_2O (65:35)	4.3
17α-Nortestosterone	CH_3OH–H_2O (65:35)	6.7
Methyltestosterone	CH_3OH–H_2O (65:35)	6.7
17β-Testosterone	CH_3OH–H_2O (65:35)	5.3
17α-Testosterone	CH_3OH–H_2O (65:35)	7.6
Boldenone	CH_3OH–H_2O (45:55)	3.2
Medroxyprogesterone	CH_3OH–H_2O (45:55)	9.0
Chlormadinone	CH_3OH–H_2O (45:55)	8.3
Megestrole	CH_3OH–H_2O (45:45)	7.4

that SPE clearly is less effective than HPLC and IAC. The suitability of SPE depends on the limit of detection needed. The sample analyzed contained *ca.* 1 μg/l, approaching the limit of detection after SPE but clearly exceeding it after HPLC or IAC. The main advantage of IAC, however, becomes clear in multi-residue analyses. Similar results to those shown here can also be obtained when 2–6 different IAC materials are combined. The use of HPLC for extract clean-up frequently results in a number of different fractions, increasing the work load for subsequent analytical steps.

2.1.3. Detection and identification

Because of the sensitivity and structure information that can be obtained with GC–MS, this is the method of choice for detection and identification or determination [14,15]. With the system described below both TMS and HFB derivatives can be analysed. Diagnostic ions are summarized in Table 3. The preferred derivative is the derivative used, unless there are strong reasons for using the other derivative, *e.g.*, in multi-residue analyses. After derivatization, the reaction mixture is evaporated to dryness under a stream of nitrogen at 50°C and the derivatized residue is dissolved in 0.025 ml of isooctane.

2.1.3.1. GC–MS analysis. The following conditions are used in GC–MS analysis: gas chromatograph, HP 5890 (Hewlett-Packard); GC column, *e.g.* Macherey–Nagel Permabond SE 52; injection, 1–5 μl, splitless, 225°C; column temperature programme, 100–280°C at 20°C/min; temperature of transfer line, 280°C; mass spectrometer, HP 5970 (Hewlett-Packard); aquisition ions, see Table 3.

The evaluation of parameters such as analytical recovery, repeatability and reproducibility are important. To assess the applicability of a method, limits of detection, determination and identification have to be known. For the methods discussed here, the limit of identification is the most important. This limit depends on a number of parameters: sample size, analytical recovery, sensitivity of the detection principle and instrument used and the quality of extract clean-up procedures. The limit of identification additionally depends on the criteria applied. For forensic purposes (reference methods) within the EEC, criteria are laid down [14,15]. For low-resolution on mass spectrometry with EI ionization, at least four diagnostic ions have to be monitored, which have to elute from the GC column simultaneously, and all four must exceed the average noise + 3 S.D. Additionally, the response ratios have to be within ±10% of the corresponding ratio as ob-

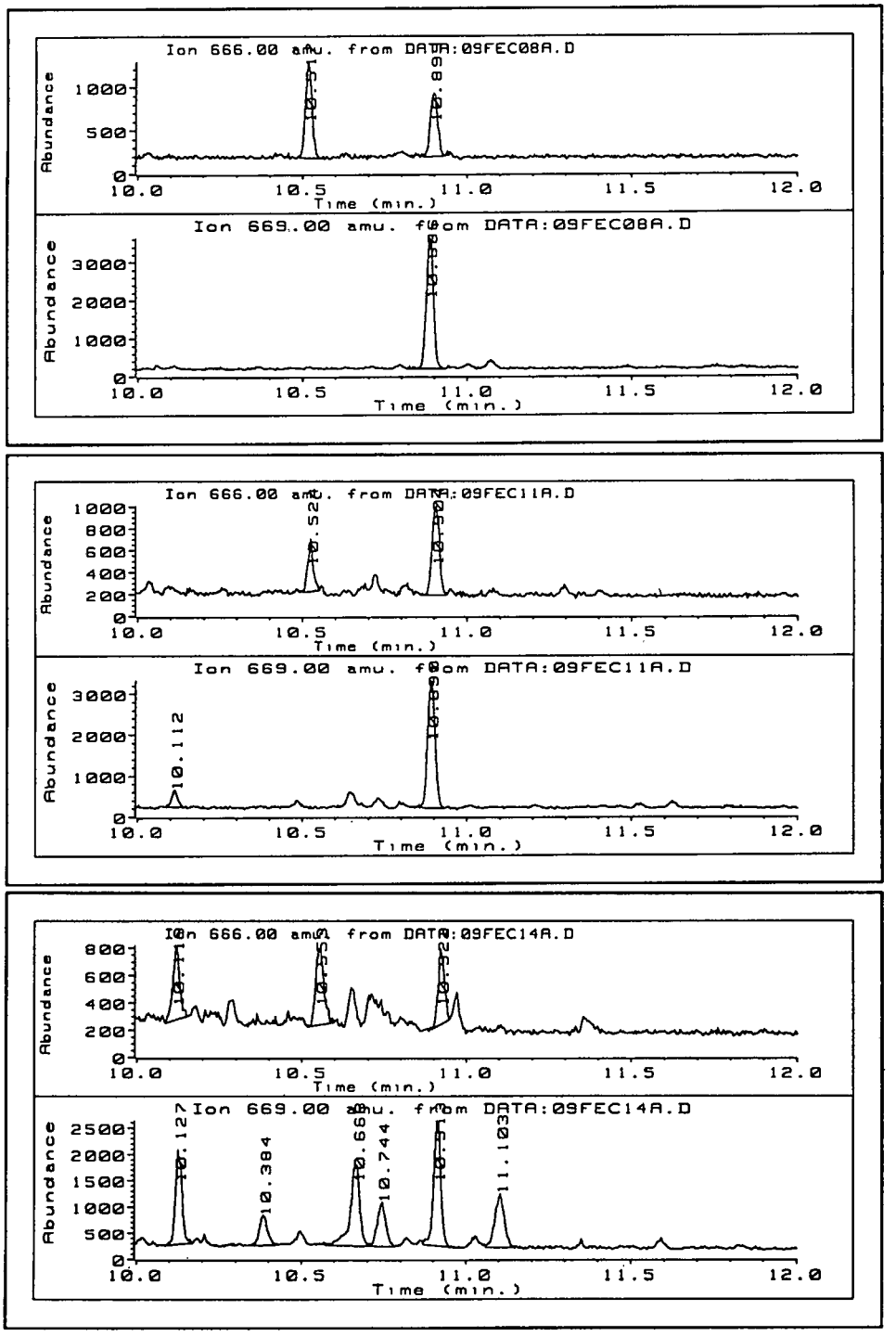

Fig. 3. Comparison of IAC (top), HPLC (middle) and SPE (bottom) for extract clean-up.

TABLE 3

IONS MONITORED DURING GC–MS ANALYSIS

The most suitable derivative is indicated by + +; (+) indicates low yield. The ion used during initial screening is given in italics.

Compound	Derivative		Ions monitored (*m/z*)
	TMS	HFB	
17β-NT	+	+ +	*660*, 453, 306, 133
17α-NT	+	+ +	*660*, 453, 306, 133
17β-T	+	+ +	*680*, 467, 355, 320
17α-T	+	+ +	*60*, 467, 355, 320
MT	(+)	+ +	480, *465*, 369, 355
Bol	+	+ +	*678*, 464, 369, 169
17β-Tb	+ +		*342*, 252, 237, 211
17α-Tb	+ +		*342*, 252, 237, 211
17α-E2	+	+ +	*664*, 451, 409, 356
EE2	+	+ +	*474*, 459, 446, 353
MPA[a]		+ +	*479*, 331, 317, 147
CMA[a]		+ +	540, *497*, 462, 401
MGA[a]		+ +	520, *477*, 421, 381
ZER	+ +	+	538, *433*, 335, 307
TAL	+ +	+	538, *433*, 335, 307
DES	+ +	+	*412*, 397, 383
DE	+ +	+	*410*, 395, 381
HEX	+ +	+	*207*, 191, 179

[a] After alkaline hydrolysis.

tained for the reference compound. For screening purposes, usually only one diagnostic ion (if possible the molecular ion) is monitored. If a sample shows a positive response it has to be reanalysed to fulfil the criteria. Usually this means that the complete procedure is repeated in which the sample is analysed in duplicate, once with and once without the addition of the internal standard because frequently deuterated analogues interfere with the detection of fragment ions owing to losses of deuterium after fragmentation. These criteria result in very different limits of identification.

Fig. 4 shows the EI-mass spectrum of DES-diTMS. The molecular ion of *m/z* 412 contains most of the response, resulting in high sensitivity (50 pg injected), which translates into a limit of detection in real samples of 0.05–0.1 μg/kg or μg/l. This low limit of detection is disadvantageous, however, for the limit of identification. The limit of identification equals the limit of detection for the ion selected in the set of four with the lowest abundance. Therefore, the limit of identification is in the region of 1 ppb, unless other ionization techniques or high-resolution instruments are used.

Fig. 5 shows the EI-mass spectrum of NT-diHFB. Here the situation is very different. The mo-

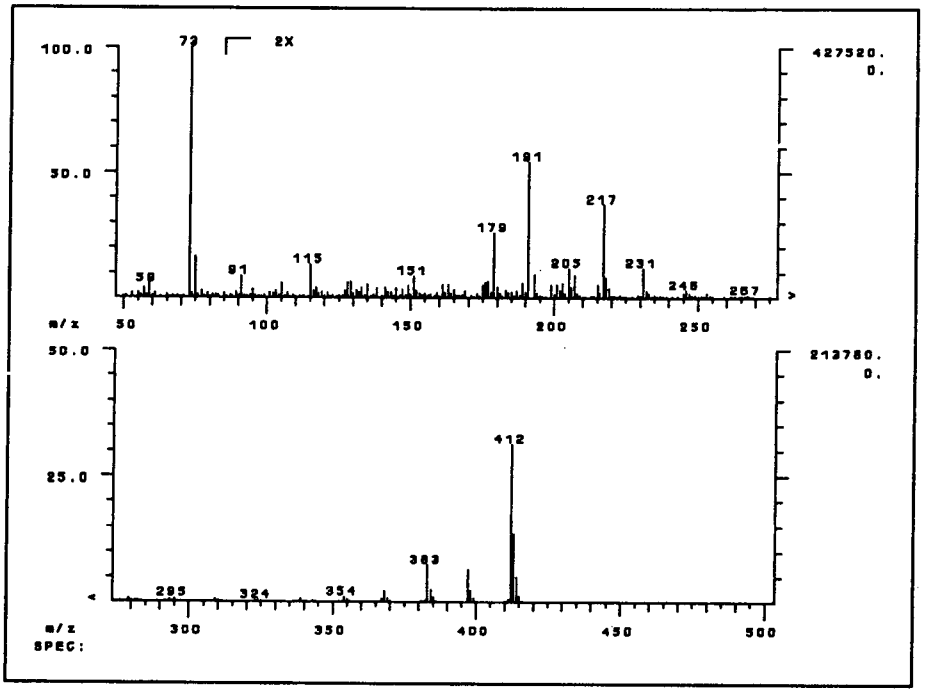

Fig. 4. Mass spectrum of DES-diTMS (EI ionization).

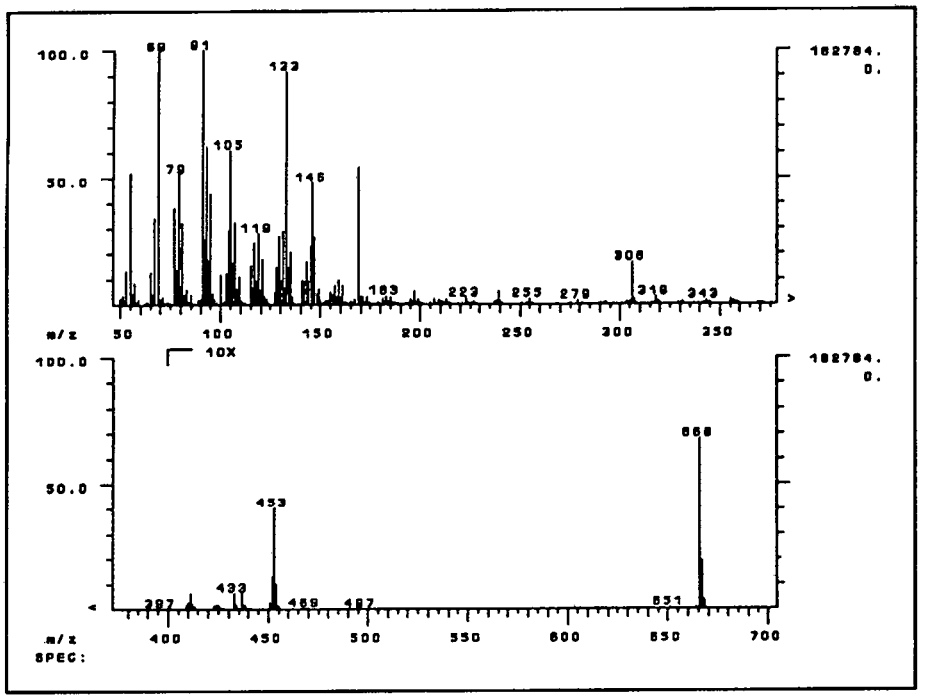

Fig. 5. Mass spectrum of NT-diHFB (EI ionization).

lecular ion of *m/z* 666 contains a much lower percentage of the total response, which is disadvantageous for the limit of detection (0.3 ppb) but favourable for the limit of identification (0.5 ppb).

The procedures described are currently in use within a number of studies, *e.g.*, national surveillance studies into the occurrence of residues, certification studies for the preparation of certified reference materials (Commission of the European Communities–BCR) and studies to determine the oral availability of (esters of) anabolic steroids. Details of the last type of studies related to nortestosterone and medroxyprogesterone will be published elsewhere in the near future. The combination of a number of different LC techniques resulted in a flexible multi-residue procedure suitable for a variety of purposes, from forensic residue analysis to toxicological studies.

2.2. Determination of β-agonists in biological materials

A shift to a relatively new class of compounds, the phenylethanolamines, primarily developed as human or veterinary drugs working on the β_2-adrenergic receptor, has been observed on the "black market" since 1988. The first of such compounds of which the large-scale misuse as a growth promoter was observed in the EEC was clenbuterol (Fig. 6) [16,17]. Within a few months in the Netherlands and some other EEC countries an effective control strategy was developed and implemented and large-scale monitoring programmes were started. However, in spite of the efficiency of the methods used and the intent of the EEC legislation, the use of β-agonists was not eradicated and a variety of related illegal alternative compounds have appeared, one of the most important being salbutamol, an out-of-patent human and veterinary drug.

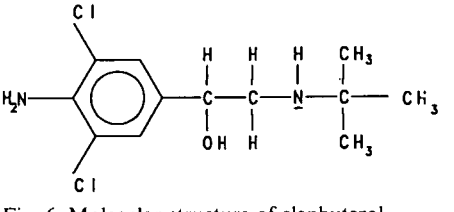

Fig. 6. Molecular structure of clenbuterol.

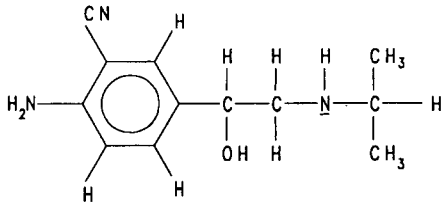

Fig. 7. Molecular structure of cimaterol.

That the use of these compounds is not without risk for the consumer recently became apparent after reports from Spain, where a serious outbreak of poisoning due to the consumption of bovine liver was demonstrated to be caused by the presence of residues of clenbuterol [18]. The majority of the β-agonists known to be used for fattening veal calves and cattle contain either an N-*tert.*-butyl or an N-isopropyl group, *e.g.*, cimaterol (Fig. 7).

A sensitive HPLC method was developed based on post column derivatization suitable for all compounds with an amine function coupled to the aromatic part of the molecule [19]. For compounds such as clenbuterol and cimaterol, very low limits of detection were achieved. An alternative procedure was developed for the simultaneous determination of compounds with either an N-*tert.*-butyl or an N-isopropylgroup. For this purpose antibodies were raised against both clenbuterol and cimaterol by coupling the amine function of the analytes to bovine serum albumin. IAC columns were prepared by mixing the individuel gels obtained by coupling the IgG fractions of the respective antisera. The columns obtained are suitable for the isolation of a large number of β-agonists. The isolated compounds are derivatized (TMS derivatives) and detected by GC–MS. For quantification and quality control, isotopically labelled internal standards are used. The method is applicable to biological matrices such as urine and liver and to animal feeds such as milk replacers and premixes. The method developed has been published in detail elsewere and has been the subject of an EEC-workshop on reference methods for β-agonists [20].

A number of studies have been undertaken to validate the analytical procedure. Most of these experiments were intra-laboratory experiments (repeatability and within-laboratory reproducilibity). In addition, the method was validated in a cooperative inter-laboratory study on animal feeds, organized by the BCR of the EEC and demonstrated during an EEC workshop in Netherlands. The conclusion of these studies was that the methodology used is suitable as a reference method.

Apart from MS, nowadays Fourier transform infrared (FT-IR) spectrometry is also a suitable identification technique in this area. Recently a new type of interfacing of capillary GC and FT-IR spectrometry has been developed. The principle of this so-called cryotraping GC–FT-IR technique (Fig. 8) is condensation of the GC eluates at 77 K on a moving infrared window with subsequent scanning of the trapped compounds by means of FT-IR microscopy [21].

A unique feature of the system is the possibility of carrying out extended post-run scanning of previously condensed compounds. As a consequence, a considerable improvement of the signal-to-noise ratio of the infrared spectra is obtained. The sensitivity is 1–2 orders of magnitude higher than that of conventional GC–FT-IR light-pipe systems and detection limits into the picogram range have been

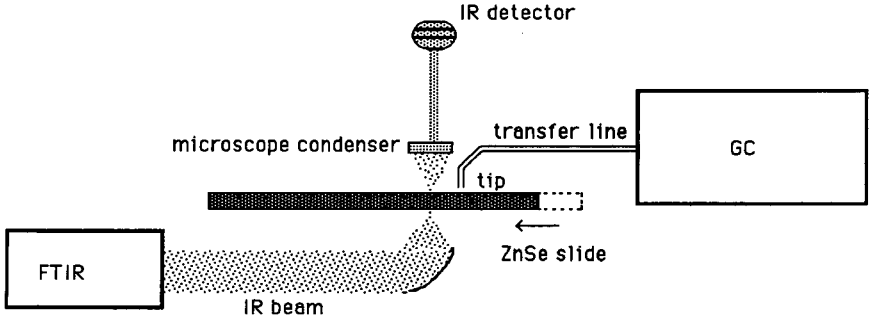

Fig. 8. Principle of cryotrapping GC–FT-IR spectrometry.

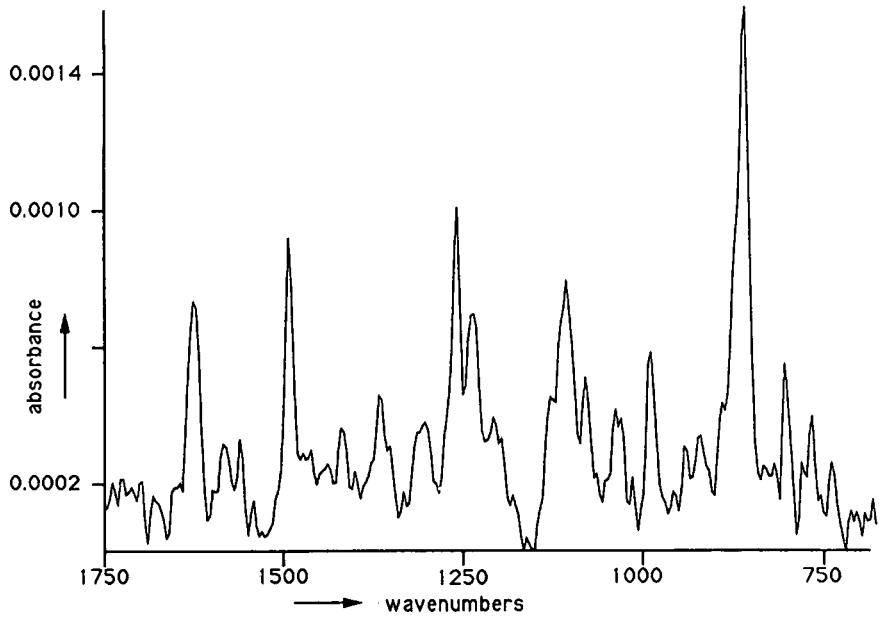

Fig. 9. Post-run spectrum (512 scans co-added) of a sample containing 5 μg/l of clenbuterol-TMS.

reported [22,23]. In view of the high discriminating properties of IR spectrometry, the technique might become a valuable tool in addition to GC–MS.

Recently we applied the technique to the identification of clenbuterol in samples of bovine urine. Fig. 9 shows a spectrum obtained for a sample containing 5 μg/l of clenbuterol. In Table 4 characteristic absorption maxima are compared for a reference spectrum, an extract of a sample of blank urine spiked at 10 μg/l and a sample obtained from a treated animal containing *ca.* 5 μg/l.

3. LC WITH ON-LINE DETECTION

The development of very sensitive UV–VIS detectors and multi-wavelength detection by the diode-array technique has provided an additional tool for the determination of selected anabolic compounds. In addition, column-switching procedures have been developed, allowing LC methods to be more specific and sensitive in combination with automation. We have developed an automated procedure for the determination of trenbolone in urine and muscle. Currently the method is used within an EEC programme on the development of certified reference materials for the determination of trenbolone in urine. For this purpose the previously de-

TABLE 4

INFRARED ABSORPTION MAXIMA (cm^{-1}) OF TMS-DERIVATIZED CLENBUTEROL OBTAINED BY CRYO-TRAPPING GC–FT-IR SPECTROMETRY

Reference	Spiked (10 μg/l)	Sample (5 μg/l)
719.5	719.42	721.0
749.9	749.8	749.3
786.2	786.5	786.4
842.0	842.0	842.2
855.1	855.6	–
905.5	906.6	907.9
926.6	926.9	928.2
976.0	975.9	977.0
1027.5	1027.2	1026.0
1067.7	1068.2	1068.6
1095.5	1094.8	1095.6
1195.3	1198.1	1199.1
1229.9	1225.7	1228.1
1250.5	1251.5	1250.5
1296.6	1298.8	1228.1
1360.8	1360.8	1360.0
1415.2	1413.2	1414.4
1488.1	1487.8	1488.6
1561.4	1555.4	1557.9
1581.6	1582.9	1580.4
1621.3	1621.2	1622.9

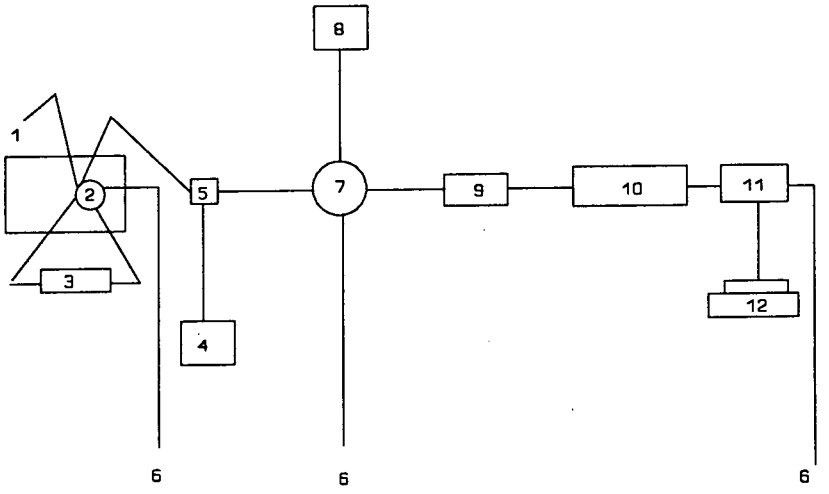

Fig. 10. Column-switching system for IAC–HPLC analyses.

scribed method [1] based on HPLC was automated using a column-switching procedure (Fig. 10).

During step 1, the enzymatically hydrolysed and centrifuged sample of urine (1) is injected directly on to an immunoaffinity column (3). The soft gel (Pharmacia Sepharose 4B Fast Flow) is packed in a glass column (Perstop; 10 × 1.2 cm I.D.) placed within the injection loop of an autoinjector (Gilson 231) (2). An aliquot of 5 ml is flushed through the column, which is subsequently washed with 10 ml of water. During step 2, the bound analytes are eluted with ethanol–water (50:50, v/v). The eluate is diluted on-line (5) with water (4) to a final ethanol content of $<10\%$ (v/v). The diluted eluate is flushed through a reversed-phase concentration column (9) on which the analytes are trapped. During this step the IAC column is in series with the concentration column and the waste (6), thus preventing the build-up of unacceptable pressure over the IAC column. During step 3, valve 7 is switched in such a way that the concentration column is placed in series with the HPLC system [LKB 2150 solvent-delivery systems and a Model 2152 LC controller (10)]. The eluted analytes are detected by measuring their UV absorbance at 350 nm (Kratos Spectroflow 773) (11). If necessary fractions can be collected (LKB Redirac) (12).

For accurate determination, *e.g.*, during homogeneity tests on lyophilized candidate reference materials, the samples are analysed in the presence of a deuterated internal standard, in our laboratory 17β-trenbolone-d_2. In samples of urine the major metabolite is 17α-trenbolone, which is completely separated during HPLC analysis, thus allowing quantification by both HPLC and GC–MS. Strictly this is a pseudo-[M]IAC application, as the antibody binds both 17α-trenbolone and 17β-trenbolone. Fig. 11 shows two chromatograms, one for a sample that does not contain trenbolone and the other from a sample obtained from an animal treated with Trenbolonacetate.

Column switching, combining IAC with reversed-phase LC, has proved to be a highly suitable technique for the determination of trenbolone in samples of urine and tissue.

4. LC–MS METHODS

Combined LC–MS is one of the most rapidly advancing coupled techniques. However, its use within the area of steroid and β-agonist analyses has so far been limited, the main reason being the availability of numerous analytical alternatives. The widespread use of GC–MS and the limited resolving power of LC were no stimulus for the development of LC–MS procedures. There are, however, a number of research applications of LC–MS which clearly demonstrate the power of this technique. LC–MS has proved to be particular interesting in metabolism and conjugation studies and in analyses of highly polar corticosteroids.

Shackleton *et al.* [24] developed a method for the

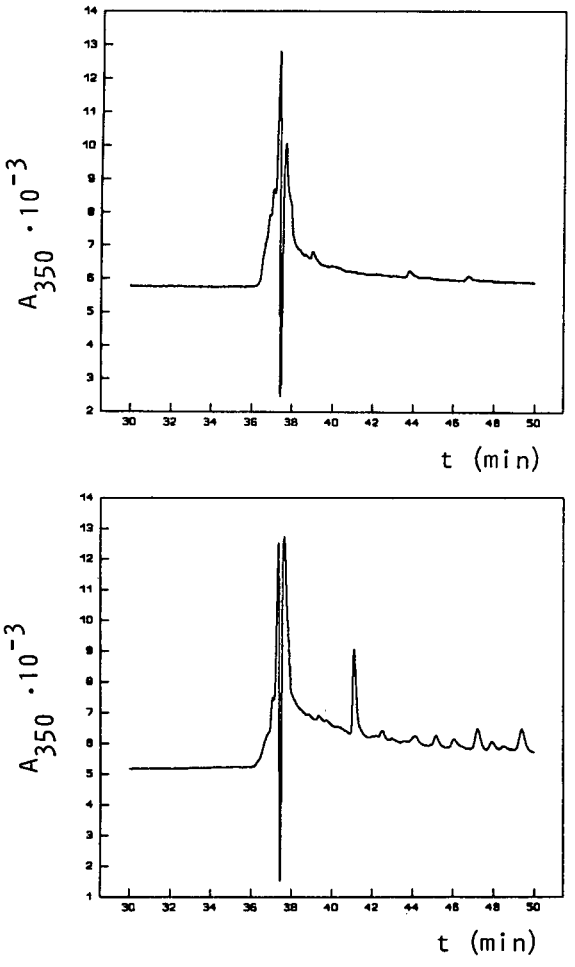

Fig. 11. HPLC separations of samples of urine obtained after on-line IAC–HPLC analysis. Upper panel: blank sample; lower panel: obtained from treated animal.

simultaneous determination of dehydroepiandrosterone sulphate, androsterone sulphate, epiandrosterone sulphate and androst-5-ene-3β,17β-diol sulphate in human serum, based on LC–isotope dilution MS. Poon *et al.* [25] demonstrated the suitability of LC–MS in a study of the metabolism of 4-hydroxyandrost-4-ene-3,17 dione in breast cancer patients. Seven metabolites were identified in samples of urine after hydrolysis, liquid–liquid extraction and reversed-phase LC–MS using a thermospray interface.

Sturm *et al.* [26] identified a number of metabolites of medroxyprogesterone acetate (MPA) in human plasma, a knowledge of which is important as these metabolites could well be responsible for the beneficial effect of MPA in breast cancer patients.

Several applications in which corticosteroids were determined using LC–thermospray MS [27–29] further illustrate the importance of LC–MS, especially in areas where GC–MS is not suitable because of the high polarity of the analytes or when derivatization should be avoided.

5. CONCLUSIONS

The current tendency within residue analysis is the development of either fast screening procedures, frequently based on an immunochemical detection principle, or methods based on GC combined with MS for confirmation. During recent years, however, the distinction between these two types of methods has become less clear because of the improved methods of sample clean-up which allow the selective isolation of groups of compounds. Mixed-mechanism SPE procedures and multi-immunoaffinity techniques are clear examples of LC developments that have greatly contributed to the current state of the art within residue analysis.

Screening methods are methods optimized for maximum sample throughput and a minimum chance of obtaining false-negative results. LC is not a preferred technique within such applications because it still is a relatively laborious procedure. For confirmation analysis, LC is frequently used. The LC–immunogram and LC–diode-array procedures are typical examples of methods used for confirmation. For this purpose the EEC has laid down criteria for positive identification [14,15] with such procedures. Apart from general criteria for assay performance, the amount and quality of information are specified.

A reference method should be able to yield direct structure information on the compound detected. For this purpose, LC has to be combined with one of the advanced spectroscopic techniques such as MS, IR spectrometry of UV–VIS spectrometry. So far MS is the only technique suitable in cases of international dispute. The role of LC, other than for puposes of sample clean-up, within such methods has been limited. LC–MS has found only a limited number of applications within residue analysis. In the near future this situation is likely to change.

LC is a key technique within residue analyses for

steroids, β-agonists and related compounds. The use of immunoaffinity chromatography, column-switching procedures and the further advancement of LC–MS will be areas of future developments.

6. ABBREVIATIONS

BCR	Community Bureau of Reference
BOL	Boldenone
CMA	Chlormadinone acetate
DE	Dienestrol
DES	Diethylstilbestrol
E2	Estradiol
EE2	Ethynylestradiol
EEC	European Economic Community
EI	Electron impact
GC	Gas chromatography
HEX	Hexestrol
HFB(A)	Heptafluorbutyric acid (anhydride)
HPLC	High-performance liquid chromatography
IAC	Immunoaffinity chromatography
ID	Isotope dilution
LC	Liquid chromatography
MGA	Megestrol acetate
MIAC	Multi-immunoaffinity chromatography
MT	Methyltestosterone
MPA	Medroxyprogesterone acetate
MS	Mass spectrometry
NT	Nortestosterone
SPE	Solid-phase extraction
T	Testosterone
TAL	Taleranol
TBME	*tert.*-Butyl methyl ether
TMS	Trimethylsilyl
Tb	Trenbolone
ZER	Zeranol

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Review

Drug residue analysis using immunoaffinity chromatography

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ABSTRACT

The background and applicability of immunoaffinity chromatographic separations and clean-up to drug residue analysis of agricultural commodities is discussed. The uses of antibody specificity for separation and concentration of drug residues are presented. Examples of immunoaffinity chromatography for the determination of residues of (1) nortestosterone and methyl testosterone in swine muscle, urine and bile; (2) chloramphenicol in swine tissue, eggs and milk; (3) clenbuterol in calf urine; (4) zeranol and β-zearalanolin in calf urine: (5) diethyl stilbesterol, dienestrol and hexestrol in calf urine are presented. Further, examples of the successful coupling of immunoaffinity separations with other chromatographic techniques such as gas chromatography and high-performance liquid chromatography are presented.

CONTENTS

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1. INTRODUCTION

Immunoaffinity chromatography (IAC), in its various forms, is a rather specialized form of affinity chromatography [1,2] wherein the separatory ligand is either an immobilized antibody or antigen. For the purposes of these discussions on drug residue analysis, the antibody will be the separatory ligand. The selective separation occurs through the classical antibody–antigen reaction

$$Ab + Ag \rightleftharpoons AbAg$$

where Ab is the antibody and Ag is the antigen and the complex formed is represented by AbAg.

IAC for antigen isolation is completely dependent upon the antibody to separate the target compound. The antibody ligand is immobilized on a support and, as the target compound comes into contact with it, a complex is formed. The ligand–target compound complex is disassociated because of hydrophobic changes caused by the mobile phase and the target compound is eluted from the column. The disassociation of the complex takes place after other materials have passed or been washed through the column. The specificity of the antibody leaves a minimum of interfering materials to be eluted from the column. Thus, the eluate can be isolated relatively pure.

2. THE ANTIBODY

The literature is replete with references to the production and purification of antibodies. Interested readers should utilize the texts and papers referenced as a start in undertaking the process of preparing antibodies [3–5]. However, regardless of which type of antibody is used, monoclonal or polyclonal, the antibody should be considered as a reagent. The antibody reagent may have different stability and handling characteristics than the standard chemical reagent, but, if the singular attributes and liabilities of the antibody are understood, the antibody should pose no greater problem of handling and use than any other delicate reagent.

2.1. Polyclonal antibodies

These antibodies are raised by immunizing animals with a specific antigen. In general, molecules with molecular masses greater than 5000 usually can elicit an antibody reaction in the animal. Molecules that have low molecular masses, such as those of drugs, usually do not stimulate an immunogenic response. Such a molecule usually must be linked to a large molecule such as a protein (bovine serum albumin, ovalbumin, human serum albumin, thyroglobulin, hemocyanin) to become immunogenic. The serum of immunized animals contains several antibodies called polyclonal because the antibodies are products of several B-cell clones. It also may be considered that the polyclonal antibody is but a mixture of monoclonal antibodies that are extremely difficult to separate.

2.2. Monoclonal antibodies

Antibodies are raised in mice via immunization with an antigen complex followed by further injections 4–6 weeks later to boost the titer of antibodies. Within a few days after the booster treatment, the spleen is removed from the mouse and the mouse lymphocytes are fused in the presence of polyethylene glycol with cultured mouse myeloma cells that are deficient in the enzyme hypoxanthine–guanine rybosyltransferase (HAT). The fused cells or heterokaryons are cultured in a medium containing HAT which prevents the growth of the myeloma cell. After the death of the mouse lymphocytes, only heterokaryon cells that posses the combined traits of both the lymphocytes and the myeloma cells (hybridomas) survive. These hybridomas are screened for the production of the specific antibody. Cells, that produce the desired antibody, are cloned to produce a cell line that will produce antibody in either cell culture systems or through the growth of ascites tumors in mice.

3. PURIFICATION OF THE ANTIBODY

The classical first step in the purification of the antibody is precipitation with ammonium sulfate. Precipitation usually occurs at concentrations of 35–40% saturation; concentrations greater than this do not improve the antibody yield and, instead, will increase contamination with other proteins. Since the presence of ammonium ions can interfere with the further use of the antibody, ammonium ions are removed commonly by dialysis. Since ammonium sulfate precipitation rarely purifies the protein, other enrichment/concentration steps are necessary.

Ion-exchange chromatography using DEAE cellulose resin is one of the basic modalities for the purification of antibody. Antibodies are basic serum proteins with isoelectric points between 6 and 8. At a pH of 8, the antibodies carry a negative charge while the DEAE cellulose has a strong positive charge. Thus, there is a binding usually at low ionic strengths. The antibodies are eluted with the increasing strength of competing anions usually provided by a gradient elution system. Because the antibodies are very basic serum proteins, they elute from the column first. Similarly, antibodies can be eluted in the order of their isoelectric point by lowering the pH of the eluent.

If greater purification of the antibody is necessary, gel permeation chromatography can be used. Gel permeation chromatography is more of an adjunct to other purification systems than a primary method. Affinity chromatography can be used as a purification technique for antibodies by immobilizing the antigen on a solid matrix such as agarose and binding the antibody from solution. Elution of the antibody from the solid phase matrix can be accomplished with a relatively small volume of eluent. A cautionary word is required. It is extremely important to be careful and not use conditions that will denature the antibody.

The reverse of the system described for the purification of antibody is fundamentally IAC. Antibodies are immobilized upon a solid matrix allowing the specificity of the antibody–antigen complex to separate the target compound. Interferences are then washed away and the relatively pure target compound is eluted in small volumes from the column [6].

4. THE ANTIBODY–ANTIGEN REACTION

The antibody–antigen reaction is best described as a bimolecular reaction

$$Ab + Ag \rightleftharpoons AbAg$$

with

$$K_a = [AbAg]/[Ab]\,[Ag]$$

where [AbAg] is the equilibrium concentration of the bound ligand complex; [Ab] is the equilibrium concentration of the free ligand and [Ag] is the equilibrium concentration of the antigen. By substituting B for [AgAb], p for the total concentration of ligand, both bound and free, and q as the total concentration of binding sites the equation becomes

$$K_a = B / [(p - B) \cdot (q - B)]$$

At equilibrium, $p - B$ becomes the concentration of the free ligand. Thus, it follows that

$$B(\text{bound ligand}) / F(\text{free ligand}) \equiv R(\text{response variable})$$

If one substitutes then, $R \equiv K_{a1} - K_{a2}$ which is the classical relationship developed by Scatchard [7]. However, this equation holds for only one species of antibody binding site. If there were a second species of antibody, the equation becomes

$$R \equiv K_{a1}\,(q_1 - B_1) = K_{a2}\,(q_2 - B_2)$$

In chromatographic assays the response variable R equals the distribution coefficient K_d, which is the ratio of matrix-bound analyte to free analyte. If this relationship is substituted into the basic equation for the retention of an analyte on chromatographic columns, where $V_c = V_0 + V_0K_d$, the relationship becomes

$$V_c = V_0 + [V_0(K_aq - K_aB)]$$

From this basic equation, Van Ginkel [8] calculated the potential efficiencies of immunoaffinity columns and showed the exquisite sensitivity possible. At very low concentrations, 10^8 M, IAC would not have the problems associated with other chromatographic interactions. Van Ginkel, demonstrated rather conclusively, the IAC would be especially effective for drug residue analysis where nanogram and picogram quantities need to be isolated and measured.

Another considereation is the affinity of the antibody. Since the immunochemical reaction is rather specific, it is not critical to have high capacities for the retention of analyte. In general, the high capacity of other chromatographic systems allows retention of more interfering compounds, with loss of the advantages of high retentive capacity. The key to the balance between the capacity necessary for the retention of analyte and the ability to reuse the column lies with the use of eluting solvents.

5. THE COLUMN

As with any column chromatographic system, the heart and soul of the separation is the column. Immunoaffinity columns that can be used with a high-performance liquid chromatographic (HPLC) system must be able to withstand the rigors of the system. Phillips [9] outlined the basic requirements for HPIAC columns. He recommended stainless steel columns, usually ranging in length from 5 to 25 cm with an internal diameter of 4.6 mm. After the packing has been prepared to contain the antibody, the column can be packed either as a slurry or dry. The pump-slurry technique uses buffers with a low salt content, such as Tris or 0.01 *M* phosphate buffer to minimize friction and denaturation of the immobilized antibody (ligand). If the solid support consists of glass beads, the packing can be freeze dried after antibody attachment and packed dry.

Attachment of the antibody to the solid support requires that the combining sites of the antibody be properly oriented to the mobile phase. The antibody should be linked by the Fc or tail portion of the antibody to a binding compound or linker molecule. To accomplish this task, reagents such as carbonyl diimidizole can be used. This reagent reacts with primary amine groups of the ligand, usually at a pH of 9, which in turn is attached to the glass beads. Aminopropyl or alkylamines can be used to react with primary amino groups; this requires either diazotization or succinimide ester modification before attachment can take place. Compounds containing carboxyl groups will react with primary amine groups on the ligand at a pH of 8 to 9. Thiol groups will react with carbonyl groups of the ligand through a carbodiimide linkage at pH 9. The use of these materials for linking the antibody (ligand) to the glass bead could result in the randomized binding of the different segments of the antibody Y rather than the Fc portion. This would result in the loss of ligand binding capacity. A completely randomized binding reaction might lead to a loss of 66% of the binding capacity.

Orginally obtained from the cell wall of *Staphylococcus aureus* and now available in recombinant form, Protein A binds antibodies through the Fc portion of the molecule. When immobilized on the glass beads, by any of the afore-mentioned systems, two of the five subunits of the Protein A will have Fc units available for attachment of antibody. The immobilized Protein A with ligand attached is now in the proper orientation. Once the antibody is attached, the other reactive sites on the glass beads are reacted with another nonreactive protein (a protein that will not react with the antigen or target compound).

Protein G, orginally derived from a streptococcus and now available as a commercial recombinant product, appears to be an excellent material for immobilizing antibodies on glass supports. It is attached to the glass in essentially the same manner as Protein A and appears to have binding qualities for many IgG antibodies that is superior to that of Protein A [9].

6. ELUTION OF TARGET COMPOUND FROM AN IMMUNOAFFINITY COLUMN

To elute the antigen from the antibody–antigen complex, the conditions on the column must be changed. The K_a must be reduced. Reduction can be accomplished by elution with a linear pH gradient or a linear chaotropic ion gradient using ions such as Cl^-, ClO_4^-, SCN^-, or CCl_3COO^-. Changing the polarity by using solvents such as methanol and ethanol or using denaturing agents such as detergents, urea or guanidine can accomplish the breaking the antibody–antigen bond, but, this could damage the antibody and limit the useful life of a column. Chaotropic agent have the tendency to reduce hydrophobic interactions and can cause some denaturation. Changing the columns temperature can also be used. As the temperature rises, antibodies have a tendency to denature. Even at room temperature, antibodies have a tendency to slowly denature. The lower the temperature the longer the life of the column and the better the peak resolution.

7. FLOW-RATES

The flow-rates govern the speed of the antibody–antigen reaction. The binding reaction is less efficient with faster flow-rates. Flow-rates between 0.4 and 4.0 ml/min are common. Optimal flow-rates are functions of the solvent systems used during the isolation of the analyte and the gradient system used for the elution. In general, it is best to determine the optimum flow-rate for each separation [9].

8. OPERATIONAL PRESSURE OF THE HIGH-PERFORMANCE IMMUNOAFFINITY CHROMATOGRAPHIC (HPIAC) COLUMN

A continuing source of concern is the operating pressure of an immunoaffinity column. Although the flows will be low in general, excessively high pressures ($>3.4 \cdot 10^6$ Pa) should not be used because the pressure will generate shear-type forces that could cause the destruction of the antibody–matrix bond and lower the efficiency of the column. In addition, it is possible to add impurities from the column material to the separated analyte. In general, pressures should be approximately $0.34 \cdot 10^6$ Pa to prevent loss of immobilized antibody.

9. SPECIFIC *VERSUS* MULTISPECIFIC ANTIBODY

Whenever the analytical problem requires the analysis of a single specific analyte and antibodies can be raised against that analyte, the analytical system is rather simple. If there are several members to a drug family, as is the case with the sulfonamide drugs, it would be logical to raise an antibody that will recognize the basic molecular structure of the drug family and use that antibody. Questions arise from this approach, namely, (a) will the multispecific antibody have sufficient affinity for all the members of the family? (b) Will the members of the family elute from the column as discrete peaks or will they come off as combined peak(s)? (c) Would a polyclonal antibody be sufficient to meet the analytical needs? (d) Would a mixture of monoclonal antibodies be required to obtain the proper separation/elution? (e) Where would metabolic products appear in this analytical scheme? (f) In multi-residue or multi-analyte analysis, would it be best to use the IAC column as a method of relatively specific separation/concentration and use more conventional HPLC procedures for separation and HPLC–mass spectrometry (MS) systems for identification?

All these questions are not totally definable at this point in time. Individual analytical situations will dictate the necessary approaches. Van Ginkel [8] discussed this problem with specific emphasis on the β-agonists and hormones and pointed out that most laboratories need multi-residue methods and that combinations of analytical separations are often necessary for completeness.

10. AVAILABILITY OF MATERIALS

With rare exception, most analytical laboratories are not equipped to produce antibodies. Nor are analytical personnel trained or desirous of producing them. IAC, in its various forms, is therefore dependent upon the commercial availability of such reagents. All too often, antibodies are proprietary products used for some other analytical purpose and are unavailable to the analytical scientist. When commercialization of antibody production for analytical purposes becomes commonplace, IAC will then realize its potential.

11. APPLICATIONS OF IAC AND HPIAC FOR RESIDUE ANALYSIS

11.1. Immunoaffinity clean up

By far the most common use of IAC for drug residues analyses has been in the area of sample clean-up. Clean-up of samples is as important to an analytical determination as any other aspect of the analytical system. Separation/concentration/purification of trace quantities of analyte (μg, ng to pg/kg) from a complex matrix is the backbone of every analytical determination. For this purpose, immunaffinity chromatography is well-suited.

A multi-immunoaffinity chromatographic column (MIAC) was used by Van Ginkel *et al.* [10] for the analysis of picogram quantities of nortestosterone and methyl testosterone in muscle. After digestion and extraction of the muscle tissue and defatting, the extract was passed through antibody-coupled tresyl-activated Sepharose column. The bound anabolic steroids were eluted with a small volume, 2 ml, of 50% ethanol. The MIAC column was regenerated with subsequent washings of 90% methanol, 0.1 *M* sodium acetate and phosphate buffer. Final determination was by gas chromatography (GC)–MS. Recoveries were 80% at levels of 0.5 μg/kg. Two aspects were especially noteworthy, namely, the ability to reuse the MIAC clean-up column at least 25 times without loss of capacity and the ability to detect and measure low levels, 0.5 μg/kg.

In a similar vein, Van Ginkel and co-workers [11–13] used a clean-up system using IAC coupled with HPLC with UV detection for residues of nortestosterone and its major metabolite in bovine urine and bile and for trenbolone and its metabo-

lite. Residues at levels of 0.1 μg/kg or less were detectable. Van Ginkel and co-workers noted that when an antibody is available, IAC surpasses many other techniques for sample clean-up.

Van de Water and Haagsma [14] used monoclonal antibody-mediated clean-up in the detection of chloramphenicol in swine muscle, milk and eggs. The skimmed milk and centrifuged egg homogenates were filtered and applied directly to the immunoaffinity column. The column was prepared by coupling the antibody to chloramphenicol to a carbonyldiimizole-activated support. No matrix interferences were noted and the recoveries, when the capacity of the column was not exceeded, was essentially 100% at spiking levels of 1 μg/kg. When a glycine–NaCl buffer was used as the eluting solvent, the immunoaffinity columns lost no binding capacity; methanol use caused a 85% loss in capacity after only 6 cycles. Van de Water and Haagsma [15,16] used a similar clean-up in a system for the detection of chloramphenicol in swine muscle tissue. The tissue was extracted with water rather than solvent and passed through a immunoaffinity column prepared by coupling antibody to CNBr-activated Sepharose. After washing, the antibody-bound chloramphenicol was eluted with methanol. After evaporation of the methanol, the residue was dissolved in mobile phase solvent and assayed by HPLC. Although the overall recoveries were 70%, the were no losses attributed to the immunoaffinity clean-up. Co-extracted meat components did not influence the immunoaffinity clean-up.

Van de Water and Haagsma [15] compared antibody-mediated clean-up (AMC) to determine residues of chloramphenicol in swine tissue and milk and compared the results with an ELISA, solid phase extraction (SPE) and a qualitative card test. Correlation was excellent between the methods. The chromatograms using the AMC were very clean with no interfering peaks.

Haasnoot *et al.* [17] used similar immunoaffinity clean-up approaches for determining clenbuterol, a β-agonist drug, in urine of calves. Similarly, H. Ong *et al.* [18] used immunoaffinity clean-up coupled with reversed-phase HPLC for the determination of albuterol, a β-adrenergic agonist in human plasma. Plasma levels of 0.79–1.56 ng/ml could be detected.

Bagnati *et al.* [19] utilized immunoaffinity separation with GC–MS of the pentafluorobenzyl ethers of zeranol and β-zearalanolin in calf urine. Levels as low as 0.17 μg zeranol and 0.24 μg zearalanolin/g were detected. Recoveries from spiked samples using the immunoaffinity columns were 84 and 64%, respectively. Urine samples containing incurred residues were treated with β-glucuronidase and arylsulfatase to hydrolyze the phase 2 metabolites (sugar or amino acid conjugates of the parent compound or some other metabolic product). Elution from the immunoaffinity column was accomplished with acetone–water (95:5, v/v). These columns were used for at least 30 analytical cycles without appreciable decline. The authors noted that similar affinity columns lasted for up to 100 cycles.

Bagnati *et al.* [20] used a very similar analytical scheme for the analysis of diethylstilbesterol, dienestrol and hexestrol residues using immunoaffinity chromatographic clean-up followed by derivatization with pentafluorobenzyl bromide and detection using gas chromatography coupled with negative ion chemical ionization mass spectrometry. The antibody used was a polyclonal raised in rabbits. Average recoveries from buffer of the afore-mentioned compounds and their isomers ranged from 42 to 92%; from urine, the recoveries ranged from 52 to 96%; from plasma, recoveries ranged from 28 to 83%. Levels as low as 0.011 μg *cis* isomer of diethylstilbesterol/kg and 0.021 μg *trans* isomer/kg were detectable. The affinity columns used were able to extract all expected compounds through the polymorphism of the polyclonal and the presence of *cis* and *trans* compounds in the immunizing complex. Noteworthy was the fact that natural estrogens were not extracted. This points to the selectivity of the immunoaffinity clean-up system.

11.2. High-performance immunoaffinity analysis

The use of HPIAC for the direct analysis of residues is far less common, in fact, there is a dearth of reports on the direct use of of HPIAC for residue analysis. Although Phillips [9] indicated that HPIAC "can be applied to the isolation of any material to which an antibody can be produced" there are some very practical reasons that direct use has not burgeoned. Elution from the immunoaffinity column may not yield sharp peaks because the desorption is slower than desirable. Thus, the resulting peak is braodened with the expected quantitation problems [21]. This phenomenon was reported by

Haagsma and Van de Water [22] in their use of this technique for the analysis of chloramphenicol residues in milk and meat. To remove the problems of peak braodening and disruption of baseline stability, Haagsma and Van de Water coupled the immunoaffinity column directly to a C_8 RP-HPLC column. The eluate from the immunoaffinity column was concentrated upon the HPLC column. The target compound, chloramphenicol was chromatographed with no reported matrix interferences at residue levels. This combined system was rugged since repeated uses, 150 samples over a 3-month period, did not result in a loss of analytical performance.

12. CONCLUSIONS

Although there are limited numbers of applications of HPIAC in drug residue analysis to report, it is apparent that immunoaffinity systems for selective clean-up, separation and concentration of target compounds in residue analysis has significant value to the food scientist, pharmacologist and regulatory official. The coupling of a immunoaffinity columns with standard HPLC columns offers the analytical scientist the best of both techniques. The commercial availability of antibodies to drugs, both monoclonal and polyclonal, will be the limiting factor in the application of this technique.

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Review

High-performance liquid chromatographic methods for the determination of sulfonamides in tissue, milk and eggs

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ABSTRACT

In the last decade, significant research has been done to improve the existing high-performance liquid chromatographic (HPLC) methods and also towards developing simple, reliable and sensitive HPLC methods for sulfonamides in meat, milk and eggs. The replacement of solvent extraction with solid-phase extraction or matrix solid-phase dispersion techniques is a step forward. Significant improvements in sensitivity have been achieved. This review concentrates on HPLC methods for the determination of sulfonamides in foods of animal origin published after 1980. The existing methods are critically evaluated and suggestions for future research are made.

CONTENTS

1. INTRODUCTION

Within the last three decades, the use of veterinary drugs in animal husbandry programs has increased substantially. Although the use of veterinary drugs has helped to increase the food supply, negative consequences, such as the presence of drug residues in foods, cannot be ignored. As the use of veterinary drugs has increased, the possibility of consumers being exposed to these drugs has also increased. The United States Food and Drug Administration (FDA) sets the tolerance levels of approved drugs in foods. As long as the residues are below the allowed tolerance limits, the food is considered safe for human consumption. Some of the food supply which reaches the consumer, however, does contain drug residues over tolerance limits.

There are two main reasons for the presence of

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TABLE 1

STRUCTURES OF SULFONAMIDES

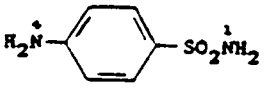
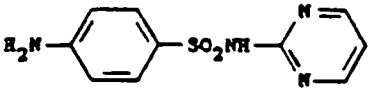
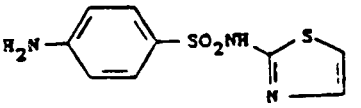
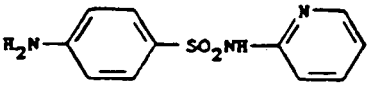
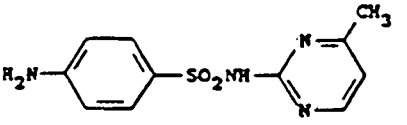
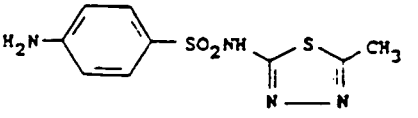
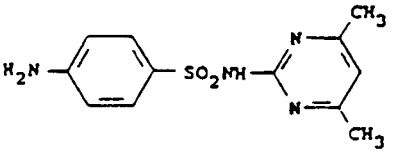
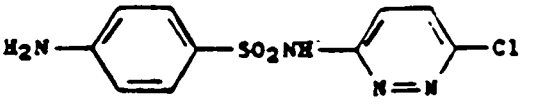
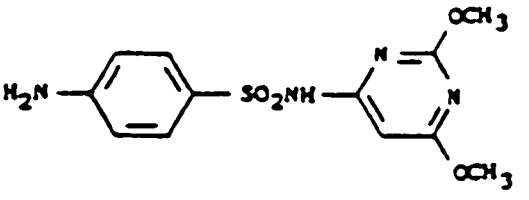
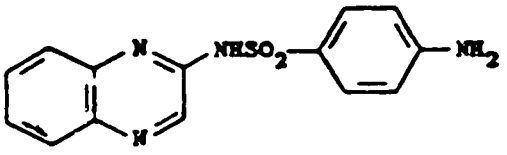
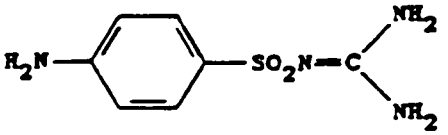
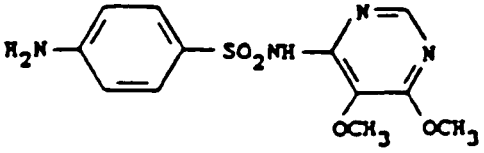
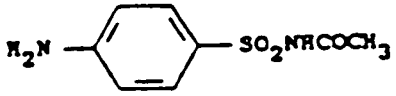
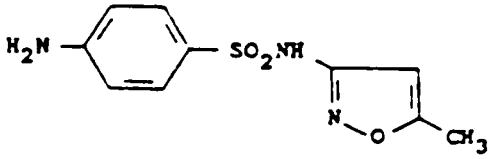
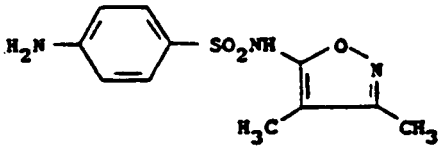
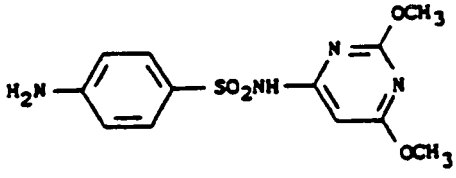
Compound	Structure	pK_a
Sulfanilamide		10.4
Sulfadiazine		6.4
Sulfathiazole		7.2
Sulfapyridine		8.56
Sulfamerazine		7.0
Sulfamethiazole		5.45
Sulfamethazine		7.5
Sulfachloropyridazine		5.1
Sulfadimethoxine		6.2

TABLE 1 (*continued*)

Compound	Structure	pK_a
Sulfaquinoxaline		5.5
Sulfaguanidine		11.3
Sulfadoxine		5.9
Sulfaacetamide		6.1
Sulfamethoxazole		5.4
Sulfisoxazole		4.79
Sulfamonomethoxine		6.5

violative residues in foods. First, improper, illegal or extra-label use of drugs can result in violative residues. Second, an insufficient withdrawal period can also cause violative residues. Every drug has a set withdrawal period before the residue levels in the animal body drops below the tolerance level. If this withdrawal period is not maintained before slaughter, higher residue level may be present in food.

The presence of drug residues in foods can be a health hazard to consumers. First, carcinogenicity of some drugs may be a serious concern. Second, continuous exposure of certain microorganisms to these drugs may result in the development of drug-resistant strains.

Sulfonamides are a class of antibacterial drugs which are used in farm animals for the treatment of a variety of bacterial infections. In food-producing animals, sulfonamides are used not only for therapeutic but also for prophylactic purposes. Chemically, sulfonamides are substituted aromatic amines substituted at the N-1 position. Table 1 shows the structures of important sulfonamides which are discussed here.

In the past, residues of sulfa drugs have been found in milk offered for sale. A nationwide survey by the FDA in 1988 reported that 45% of milk samples contained detectable amounts of sulfamethazine [1]. Another survey of 30 samples, done in ten cities across Canada, found sulfamethazine residues in two samples at levels of 11.40 and 5.24 ppb [2].

After a report from the National Center for Toxicological Research (NCTR) in 1988 indicating that sulfamethazine may be a carcinogen, concern over the presence of residue of sulfamethazine and other sulfonamides in milk and other foods has grown [3]. The FDA has, therefore, set tolerance limits of sulfonamides in meat, milk and poultry (Table 2). Currently, sulfadimethoxine is the only sulfa drug allowed for use in lactating animals, and the residue may not exceed 10 ppb in milk [4]. In meat and poultry, residues of sulfonamides may not exceed 100 ppb [4].

This review concentrates on high-performance liquid chromatographic (HPLC) methods for the determination of sulfonamides in foods of animal origin. The existing methods are critically evaluated and suggestions for future research are made.

TABLE 2

FDA TOLERANCES FOR SULFONAMIDES IN FOODS OF ANIMAL ORIGIN

Other sulfonamides not included in the table have a zero tolerance.

Drug	Tolerance (ppb)			
	Cattle	Swine	Poultry	Milk
Sulfamethazine	100	100	100	–
Sulfathiazole	–	100	–	–
Sulfadimethoxine	100	–	100	10
Sulfachloropyridazine	100	100	–	–

2. METHODS OF ANALYSIS

Owing to the concern over residues of sulfonamides in food products of animal origin, a number of techniques have been proposed for their detection, including, microbiological [5,6], immunoassay [7], thin-layer chromatography (TLC) [8–14], gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) [15–24] and HPLC [25–38].

2.1. Microbiological and immunoassay

Microbiological and immunoassay methods [5–7] are not considered very specific and may give false-positive results. These methods can be applied to liquid samples directly, but for tissue samples the tissue must be extracted to isolate drug residues before applying these tests.

2.2. Thin-layer chromatography

TLC [8–14] has been used for the detection of sulfa drug residues in foods, but these methods have limited application and are generally used only for screening or qualitative analysis. The method of Clark *et al.* [14] can screen for eight sulfonamides in milk at a low level of 10 ppb. This method is being used by the FDA as a rapid screening method, which must be followed by an HPLC method for quantitative analysis.

2.3. Gas chromatography

GC methods [15–24] have not gained wide acceptance in spite of being very sensitive and specific. In general, GC methods require solvent extraction

followed by a sample clean-up step. It is essential to derivatize sulfonamides to prepare a volatile derivative before GC analysis. The derivatization generally involves either N-methylation or N-methylation followed by acylation of the N^4-primary amino function with pentafluoroalkane carboxylic anhydride [23].

2.4. High-performance liquid chromatography

HPLC has become the most widely used technique for the determination of sulfa drug residues in meat, milk and poultry, and a number of methods have been published. Table 3 summarizes the HPLC methods which are reviewed here.

3. HPLC METHODOLOGY

A major problem in developing methodology for drug residue analysis is the detection of these drugs in biological matrices such as meat, milk and eggs at low ppb levels in the presence of potentially interfering compounds. A general approach for the determination of sulfonamide residues in foods of animal origin involves extraction, sample clean-up and HPLC analysis steps. A critical and extensive review of the methodologies developed up to 1980 was published by Horowitz [39,40]. Therefore, only methods reported after 1980 are reviewed here.

3.1. Extraction

Traditionally, the extraction of sulfonamide from meat, milk and eggs has been done with organic solvents. The use of large amounts of organic solvents makes these methods very laborious and time consuming. Sulfonamides are not very soluble in non-polar solvents, but have good solubility in polar solvents. Therefore, the extraction is generally done with chloroform, methylene chloride, acetone, acetonitrile or ethyl acetate. Some organic solvents also denature the sample protein, which results in a cleaner extract and also helps in extracting the drug residues bound to proteins. Some of the newer methods have replaced the traditional liquid extraction step with solid-phase extraction (SPE), which eliminate the use of large amounts of solvent for extraction.

3.2. Sample clean-up

Sample clean-up step is performed by back-extraction of sulfonamides into an aqueous medium. When extracting from an organic into an aqueous phase, the adjustment of the pH of the aqueous phase is critical to obtain complete recoveries. SPE has also been used for sample clean-up in newer methods. Depending on the sample matrix, sometimes a few more clean-up steps are needed before HPLC analysis. The purpose of these additional clean-up steps is to extract sulfonamides selectively while leaving other interfering compounds behind.

3.3. HPLC analysis

After the sample has been cleaned up, the HPLC analysis is performed to identify and determine sulfonamides. In general, a reversed-phase HPLC column is used with a mobile phase consisting of buffer and organic solvent (methanol or acetonitrile). For detection, either variable-wavelength UV or UV–VIS photodiode-array detectors are used. In some instances sulfonamides are derivatized and a fluorescence detector is used.

4. METHOD DEVELOPMENT AND EVALUATION CRITERIA

An analytical method must meet the criteria of evaluation, which is primarily based on its precision, specificity, accuracy and practicability. Precision is a measure of repeatability and reproducibility of the method. Repeatability indicates the variability within the laboratory or the variability of the analyst. Reproducibility indicates the variability between laboratories. The specificity of the method means that under the conditions used, no other compound (from matrix of potential external source) should show a response that may interfere with the analysis. The specificity of the method may be proven by spiking the sample with potential interferents and analyzing by the proposed method. Accuracy of the method is determined by spiking the blank sample with known amounts of standards at various concentrations and recovering the parent compound and their metabolites, if any. It is emphasized that the method development should also include incurred samples as the method may perform differently on an incurred sample than in a spiked sample. Finally, the method should be practical, which is judged on the basis of total operational requirements of the method such as chem-

TABLE 3

HPLC METHODS FOR THE DETERMINATION OF SULFONAMIDES IN MEAT, MILK AND EGGS

Abbreviations: SNL = sulfanilamide, SDZ = sulfadizine, STZ = sulfathiazole, SPD = sulfapyridine, SMR = sulfamerazine, SMTZ = sulfamethiazole, SMZ = sulfamethazine, SCP = sulfachloropyridazine, SDM = sulfadimethoxine, SQX = sulfaquinoxaline, SG = sulfaguanidine, SMX = sulfamethoxazole, SDX = sulfadoxine, SAA = sulfaacetamide, STX = sulfatroxazole, SFZ sulfisoxazole, SMM = sulfamonomethoxine.

Sulfonamide	Sample matrix	Sample preparation	Mobile phase	Column	Detection	Detection limit (ppb)	Ref.
SDZ, SMZ	Milk	Extraction with chloroform	Water–methanol (75:25)	RP-C_2, 10 μm, 250 × 4.6 mm I.D.	UV, 263 nm, electrochemical	10	Alawi and Rüssel [25]
27 Sulfonamides	Milk, meats eggs	Deproteination with acetonitrile. Extraction with hexane. Extraction with methylene chloride. Partition between hexane and aq. methanol. Extraction with ethyl acetate	Sodium acetate–CH_3CN	Spherisorb ODS, 5 μm, 250 × 4.6 mm I.D.	UV, 290, 310 nm	20–50	Malisch [26]
SDZ, SMR, SDM, SQX, SMX, SDX, SMZ, SCP, SAA, STX, STZ, SNL, SG	Milk, meat eggs	Dialysis with cellulose membrane. Enrichment on Bondapak C_{18}–Corasil, XAD-2 or XAD-4 column	Sodium acetate–CH_3CN	LiChrosorb RP-8, 10 μm' 250 × 4.6 mm I.D. CpTM-Sphere C_{18}, 7 μm, 250 × 4.6 mm I.D. μBondapak C_{18}, 10 μm, 300 × 3.9 mm I.D.	UV, 280 nm or fluorescence, 450 nm, after postcolumn deriv. with DMAB	10	Aerts *et al.* [27]
SDZ, SMR, SMZ, SMX, SQX	Milk, meats eggs	Extraction with acetonitrile. Water removal with methylene chloride. Dissolution in methanol–buffer. Extraction with hexane	Sodium acetate–CH_3CN	MOS Hypersil, 3 μm, 120 × 4.6 mm I.D.	UV, 275 nm	100	Petz [28]
SMZ	Milk	SPE extraction with C_{18}, acidic alumina and ion exchange	Potassium phosphate–methanol	LC-18, 5 μm, 250 × 4.6 mm I.D.	UV, 270 nm	0.5	Unruh *et al.* [29]

STZ, SDZ, SMR, SMZ, SMX, SDM, SFZ	Infant formula	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosphoric acid–CH_3CN	MCH-10, 300 × 4 mm I.D.	Photodiode, 270 nm	62.5	Long *et al.* [30]
SNL, STZ, SDZ, SMR, SMZ, SMX, SFZ, SDM	Milk	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosporic acid–CH_3CN	Supelcosil LC-18, 3 μm, 75 × 4 mm I.D.	Photodiode, 270 nm	31.2	Long *et al.* [31]
SNL, STZ, SDZ, SMR, SMZ, SMX, SFZ, SDM	Pork tissue	MSPD extraction with C_{18} material. Elution with methylene chloride	Phosporic acid–CH_3CN	MCH-10, 300 × 4 mm I.D.	Photodiode, 270 nm	31.2	Long *et al.* [32]
SNL, SMZ, SD, SQX, SDX	Swine tissue	Extraction with chloroform–acetone. Cation exchange with aromatic sulfonic acid column. Treatment with ammonia. Elution with methanol	Ammonium acetate–CH_3CN	Spherisorb C_8, 250 × 4.6 mm I.D.	UV, 254 nm	50	Haagsma and De Water [33]
SMZ, SDM, SMM	Tissue, eggs	Extraction with acetonitrile. Extraction with hexane to remove lipids. SPE with Bond-Elut C_{18}. Elution with 0.1% TEA–CH_3CN	Potassium phosphate–CH_3CN	Nucleosil 100 C_{18}, 5 μm, 250 × 4.6 mm I.D.	UV, 268 nm	20–40	Horie *et al.* [34]
SMZ	Milk	Extraction with chloroform. Partition between potassium phosphate buffer and hexane	Potassium phosphate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Weber and Smedley [35]
SNL, SDZ, STZ, SPD, SMR, SMZ, SMTZ, SCP, SDM, SQX	Milk	Extraction with chloroform–acetone. Partition between potassium phosphate buffer and hexane	Potassium phosphate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Smedley and Weber [36]

(Continued on p. 418)

TABLE 3 (*continued*)

Sulfonamide	Sample matrix	Sample preparation	Mobile phase	Column	Detection	Detection limit (ppb)	Ref.
SMZ		Extraction with chloroform. Partition between potassium phosphate buffer and hexane. SPE extraction with Cyclobond-I column	Ammonium acetate–methanol	LC-18-DB, 5 μm, 250 × 4.6 mm I.D.	UV, 265 nm	5	Agarwal [37]
SDZ, STZ, SPD, SMR, SMZ, SCP, SMTZ, SDM, SQX		Extraction with chloroform. Partition between potassium phosphate buffer and hexane. SPE extraction with Cyclobond-I column	Ammonium acetate–methanol	LC-18-DB, 5 μm, 250 × 2 mm I.D.	UV, 265 nm	10	Agarwal [38]

icals, instrumentation and time required for completion of the analysis. A good method may not meet al the criteria, however, as discussed above.

As the number of drugs being used on animals is increasing, the methods developed for individual drugs are becoming unattractive, and efforts are being concentrated towards developing multi-residue methods. In the case of sulfonamides, several multi-residue methods have been developed which can determine a whole range of sulfonamides simultaneously.

The detection limit of the method is another important aspect. The method should be able to determine the residue below, or at, the allowed tolerance levels. For sulfonamides, as indicated earlier, the tolerance limit is 10 ppb in milk and 100 ppb in meat and poultry. Unless a method can achieve such detection limits, it is not considered useful for regulatory purposes for a given sample type.

5. HPLC METHODS FOR THE DETERMINATION OF SULFONAMIDES

The method reported by Alawi and Rüssel [25] is applicable to milk. This method requires a simple chloroform extraction followed by HPLC analysis. Electrochemical detection was found to be more selective and sensitive than UV detection. When a UV detector was used at 263 nm the peaks co-eluting with sulfonamides interfered with the analysis. Electrochemical detection was more specific for sulfonamides and the co-eluting peak did not show any response to the electrochemical detector. An RP-C2 column was used for HPLC analysis. The mobile phase was methanol–water (25:75) containing 0.01 *M* $LiClO_4$ as an electrolyte. This method, however, is applicable only to sulfadiazine and sulfamethazine down to a low level of 10 ppb.

A method reported by Malisch [26], which requires very extensive clean-up steps, was applied to 27 sulfonamides in meat, milk and eggs. Milk was deproteinated with acetonitrile and the aqueous acetonitrile phase was extracted with hexane to remove lipid material. The aqueous acetonitrile phase was saturated with sodium chloride and extracted with methylene chloride. After evaporating the methylene chloride extract to dryness on a rotary evaporator, the residue was further purified by partitioning between hexane and aqueous methanol. The hexane phase was discarded and the aqueous methanol extract was evaporated on a rotary evaporator to remove methanol. The concentrated aqueous extract was diluted with water and extracted twice with ethyl acetate. The combined ethyl acetate extract was evaporated to dryness and the residue dissolved in an aqueous acetonitrile–methanol for HPLC analysis. HPLC analysis was done using a reversed-phase Spherisorb ODS column with sodium acetate–acetonitrile as the mobile phase and UV detection at 290 and 310 nm. The detection limits of sulfonamides varied from 20 to 50 ppb. This method is certainly advantageous as it can determine several sulfonamides simultaneously. The lengthy extraction procedure, however, makes it difficult to use this method as a routine method and to obtain consistent recoveries.

Aerts *et al.* [27] developed an HPLC method for the detection of thirteen sulfonamides in meat, milk and eggs at a low level of 10 ppb. They explored a number of options for the clean-up and HPLC analysis and a detailed comparison was made. The fat was removed from the milk and then diluted with saline solution. Eggs were directly diluted with saline solution and meats were homogenized with saline solution. Various concentrations of sodium azide solution were added to these saline solutions as an antioxidant.

Samples were dialyzed using a flat cellulose membrane to separate drugs from larger proteins or lipid molecules. The aqueous dialysate containing sulfa drugs was loaded on a short stainless-steel column which served as a concentrator. A variety of packing materials were tested, including Bondapak C_{18}-Corasil, Perisorb KAT, Perisorb RP-2, XAD-2, XAD-4 and Baker-C_8. With respect to retention and elution of sulfonamides, XAD-2 and XAD-4 were excellent but the retention was not very specific to sulfonamides as numerous UV-absorbing compounds present in the sample matrix were also retained on these phases and were eluted with sulfonamides. Bondapak C_{18}-Corasil gave very good results with milk samples but meat and egg samples were not cleaned up enough on this packing material. After concentration, the sample was backflushed to an analytical reversed-phase HPLC column and chromatographed with an appropriate mobile phase. Three different HPLC columns were examined; LiChrosorb RP-8, CpTM-Sphere C_{18} and

μBondapak C_{18}. The capacity factors (k') on CpTM-Sphere were highest, LiChrosorb RP-8 showed intermediate and μBondapak C_{18} the lowest k' values. The mobile phases, which contained sodium acetate–acetonitrile, were able to separate all sulfonamides on LiChrospher and CP TM-Sphere columns. The retention behaviour of sulfonamides was dependent not only on the polarity, but also on the ionization of sulfonamides. Therefore, the pH of the mobile phase also played an important role in the chromatographic separation. Direct UV detection at 280 nm could detect sulfonamide in milk but the chromatograms of meat and egg samples were not clean. Further clean-up of these samples was necessary before HPLC analysis. Aerts *et al.* [27] used a more specific detection approach to eliminate interferences instead of further clean-up. Postcolumn derivatization with *p*-dimethylaminobenzaldehyde (DMAB) and fluorescence detection at 450 nm were specific for sulfonamides and eliminated the interferences. Postcolumn derivatization not only improved the specificity but also the overall response by approximately 1.5 times. Based on the sample matrix and the level of sulfonamide residues present, the specific enrichment column, the HPLC column and the detection system can all be selected to determine all the sulfonamides in a single chromatographic run.

The method developed by Petz [28] for the detection of five sulfonamides in meat, milk and eggs has a detection limit of only 100 ppb. Milk was extracted with acetonitrile and co-extracted water was removed by the addition of sodium chloride and dichloromethane. The organic phase, which contained sulfonamides, was evaporated to dryness and the residue dissolved in a mixture of methanol and mobile phase. The extract in the methanol–mobile phase was washed with hexane to remove lipids, and then analyzed by reversed-phase HPLC with UV detection at 275 nm. A reversed-phase MOS-Hypersil column and with sodium acetate buffer (0.01 *M*, pH 4.6)–acetonitrile (75:25) as the mobile phase were used. This method is a simple approach for the determination of five sulfonamides without extensive clean-up. This method, however, is not suitable for milk samples owing to the detection limit of only 100 ppb.

A method developed by Unruh *et al.* [29] for the determination of sulfamethazine in milk at a low level of 0.5 ppb uses an SPE technique followed by either TLC or HPLC. Milk was diluted with phosphate buffer (pH 5.7) and passed through a C_{18} SPE column. Sulfamethazine, which was retained on the SPE column, was eluted with methanol. The eluent was passed through a small acidic alumina column and the eluent from this column was loaded directly on an ion-exchange column (AGMP-1). Sulfamethazine, retained on the AGMP-1 column, was then eluted with methanol–acetic acid–acetone (1:5:94) and analyzed by TLC with fluorescamine derivatization. For confirmation, HPLC analysis was done using a Supelco LC-18 column with a mobile phase consisting of 0.05 *M* K_2HPO_4 (pH 6.0)–methanol (65:35) and UV detection at 270 nm. Before loading the milk on the C_{18} SPE column, it was necessary to adjust the pH to 5.9. This serves two purposes: first, better retention of sulfamethazine was achieved at pH 5.9 than at pH 7, and second, milk was diluted and a better flow through the C_{18} SPE column was achieved. The eluate obtained from the C_{18} SPE column contained not only sulfamethazine but also other components, *e.g.*, lipids and riboflavin from the sample matrix which interfered with chromatographic analysis. These interferences were removed by using alumina and AGMP-1 resin columns. This method can determine sulfamethazine at a low level of 0.5 ppb. The use of HPLC was optional if further confirmation was required. This method is the first method that has such a low detection limit of 0.5 ppb. Introduction of SPE replaced the traditional solvent extraction step and thus made this method very convenient and attractive. This method has great potential for application to multi-residue determination of sulfonamides in milk and other foods of animal origin.

Long and co-workers [30–32] developed three methods which are applicable to the determination of eight sulfonamides in milk, infant formula and pork tissue, using a new technique, known as matrix solid-phase dispersion (MSPD), which was developed by Barker *et al.* [41]. This technique is based on the principle of surfactant or detergent use for disrupting the cell membrane of biological matrices by solubilizing the membrane components.

In the MSPD technique, milk, tissue or infant formula was blended with C_{18} packing material using gentle mechanical force. The C_{18} packing mate-

rial, which contains a C_{18} polymer phase bound to a silica support, works as a lipid-solubilizing material. This results in the disruption of the cell membrane of the sample, making the cell contents accessible for extraction. The polymer–sample matrix prepared in this manner was loaded on a column and washed with hexane to remove lipid materials. Sulfonamides which were more polar remained on the column and were eluted with methylene chloride. The eluate was analyzed by HPLC using a photodiode-array detector at 270 nm. The extracts from pork tissue and infant formula were analyzed by HPLC using an MCH-10 ODS HPLC column and phosphoric acid–acetonitrile (70:30) as the mobile phase. For milk, a Supelcosil LC-18 HPLC column with phosphoric acid–acetonitrile as mobile phase was used. The detection limits of these methods were 31.2 ppb for tissue and milk samples and 62.5 ppb for infant formula. Eight sulfomamides were determined in tissue and milk. Only seven sulfonamides were determined in infant formula. The mobile phase for infant formula contained a higher concentration of acetonitrile and sulfanilamide therefore could not be determined, as it eluted very early and was not separated from earlier eluting sample matrix peaks.

The MSPD technique simplifies the overall methodology and removes most of the interfering components from milk, tissue and infant formula without using solvent extraction [30–32]. The problem of the detection limits in milk and infant formula remains. A large sample volume with the same amount of C_{18} material could not be used as the polymer–sample matrix obtained was not dry enough for further analysis. Therefore, larger amounts of C_{18} material were required when larger volumes of milk were used. Further work is needed in this area to improve the detection limits. The MSPD technique, however, is a significant improvement over other existing methods and opens up a new direction in drug residue analysis. The only drawback of this method is the sensitivity for liquid samples, which needs to be improved.

Haagsma and De Water [33] developed a multiresidue method for five sulfonamides in tissue. The tissue sample was extracted with chloroform–acetone. It was necessary to adjust the pH of the sample to 5.5 before extraction, otherwise the recoveries were low. The recoveries from kidney tissue were still not very reproducible by this method. Clean-up was done by using an aromatic sulfonic acid cation-exchange column. All the sulfonamides were retained on the ion-exchange column when the acidified extract of tissue was passed through the column. The column was then treated with ammonia vapor and sulfonamides were eluted with methanol. Sulfonamides could also be eluted from the column with an alkaline buffer, but the eluate was not suitable for HPLC. HPLC analysis was then carried out on a Chrompack Spherisorb C_8 HPLC column with ammonium acetate (pH 4.6)–acetonitrile as mobile phase and UV detection at 254 nm. This method has a detection limit of 50 ppb and is applicable only to swine tissue.

Horie *et al.* [34] developed a method for determination of three sulfonamides in animal tissue and eggs. Extraction was done with acetonitrile, and the acetonitrile extract was further extracted with hexane to remove lipid materials. Further clean-up was done using a Bond-Elut C_{18} SPE column. It was necessary to adjust the pH of the extract to a low pH of 1–2 before loading on to a C_{18} SPE column. Excellent retention of all three sulfonamides was achieved. Elution of sulfonamides from C_{18} SPE column could be done with acetonitrile, but a larger volume was required. Use of 0.1% TEA in acetonitrile eluted sulfonamides very efficiently because the amine modifier competes with unbound silanols on the silica substrate. The eluate was evaporated to dryness and the residue dissolved in in 10 m*M* potassium dihydrogenphosphate solution. HPLC analysis was done on a Nucleosil 100 C_{18} column with potassium phosphate–acetonitrile as the mobile phase and UV detection at 268 nm. A column temperature of 40°C rather than ambient temperature resulted in better peak shapes. The detection limit of this method was 20 ppb for sulfamethazine and sulfamonomethoxine and 40 ppb for sulfadimethoxine.

A method developed by Weber and Smedley [35] for the detection of sulfamethazine in milk at a low level of 5 ppb, involving a simple chloroform extraction followed by partitioning between potassium phosphate buffer and hexane to remove lipids. The aqueous buffer phase containing sulfonamides was then analyzed by HPLC with UV detection at 265 nm. A Supelco LC-18-DB HPLC column with potassium phosphate–methanol was used as the

mobile phase. An extension of this work by Smedley and Weber [36] has been applied to the detection of ten sulfonamides in milk at a low level of 10 ppb and is currently being used by the FDA for testing milk. This method involves a chloroform–acetone extraction followed by partitioning between hexane and potassium phosphate buffer. Sulfonamides, dissolved in buffer, were then determined by HPLC. A Supelco LC-18-DB HPLC column with potassium phosphate–methanol as mobile phase was used. This method, although a simple approach for the determination of ten sulfonamides in milk, does have certain drawbacks. The method requires two different sets of HPLC conditions to determine ten sulfonamides. For this, either two HPLC systems are required, or all the samples are first analyzed for seven sulfonamides, and then the HPLC conditions are changed to analyze for the remaining three sulfonamides. It was also necessary to clean up the column after a few runs in order to avoid interference with any later eluting peaks. Additionally, a number of extraneous peaks were present in the LC trace which could make quantification difficult for earlier eluting sulfonamides.

A method developed by Agarwal [37], for the detection of sulfamethazine in milk involves SPE. Milk was extracted with chloroform, the extract evaporated to dryness and the residue dissolved in potassium phosphate buffer (pH 5.0). The extract in buffer was then passed through a Cyclobond I SPE column, which was washed with potassium phosphate buffer and then sulfamethazine was eluted with aqueous methanol and determined by HPLC with UV detection at 265 nm. A Supelco LC-18-DB HPLC column with ammonium acetate–methanol as mobile phase was used. This method has a detection limit of 5 ppb for sulfamethazine in milk.

The same technique was also used for the determination of nine sulfonamides in milk [38]. Milk was extracted with chloroform–acetone and the extract evaporated to dryness. The concentrated extract was then partitioned between hexane and 1 *M* potassium phosphate buffer (pH 4.4). The aqueous buffer, containing sulfonamides, was passed through a Cyclobond-I SPE cartridge. The sulfonamides, retained on the cartridge, were eluted with aqueous acetonitrile. Acetonitrile was removed from the eluate and the latter diluted with ammonium acetate buffer. HPLC analysis was done using a Supelco LC-18-DB microbore column and a gradient mobile phase. Use of a microbore HPLC column enhanced the sensitivity for the detection of sulfonamides. The mobile phase consisted of (A) 25 m*M* ammonium acetate buffer (pH 4.6)–methanol (850:150) and (B) 25 m*M* ammonium acetate buffer (pH 8.0)–methanol (700:300), with a gradient from 0 to 100%B in 30 minutes. All nine sulfonamides were eluted in 30 min and the column could be equilibrated to the initial conditions within the next 20 min. Use of a gradient also increased the peak resolution.

In this method a Cyclobond-I column was selected for SPE owing to the selective retention of sulfonamides on these columns. Cyclobond-I SPE columns contain β-cyclodextrin bonded to silica. Cyclodextrins are D-(+)-glucopyranose units connected by α-(1,4)-bonds to form cyclic oligosaccharides [42]. The glucose units are arranged in a fashion to form a truncated cone shape. The orientation of glucose units is such that there are no hydroxyl groups on the interior of the cavity, making it hydrophobic [43]. Formation of an inclusion complex between sulfonamides and β-cyclodextrin has been reported [44]. A detailed study to optimize the conditions for the retention of sulfonamides on the Cyclobond-I SPE column was done [45]. As sulfonamides are ionic in nature, their retention on Cyclobond-I SPE columns is pH dependent. The first ten sulfonamides listed in Table 1, which were used in this study, have a pK_a range from 5.5 to 10.4. With the exception of sulfanilamide, all nine sulfonamides were retained within the pH range 4.0–5.5. The effect of pH was most pronounced with sulfamethazine and sulfamethiazole. Sulfamethazine gave maximum retention towards higher pH (5.5) whereas sulfamethiazole gave the maximum retention at lower pH (4.0). The optimum pH of the buffer was chosen was 4.4, at which all sulfonamides except sulfanilamide gave excellent retention. This method is a significant improvement over the method of Smedley and Weber [36] and has overcome the shortcomings of that method. The drawback of this method is that it requires solvent extraction. Research is in progress in our laboratory to eliminate the solvent extraction step.

6. CONCLUSION

In the last decade, research has been carried out to improve the existing HPLC methods and also towards developing simple, more reproducible, reliable and sensitive HPLC methods for the determination of sulfonamides in meat, milk and eggs. The replacement of solvent extraction with SPE or MSPD techniques is a step forward. A significant improvement in the sensitivity of the methods has been achieved. None of these methods, which appear to be simplified and precise, have been subjected to collaborative studies. Therefore, the ruggedness and practicability of these methods have not yet been proven.

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Review

Methods for the determination of sulphonamides in meat

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ABSTRACT

Sulphonamides, due to their important antibacterial effects, are widely used in veterinary practice and animal husbandry. Residues arising from administration without observing withdrawal time sufficiently are normally the parent compounds and the N^4-acetyl derivatives, the latter being hydrolyzed to the parent compounds only during extraction under acidic conditions. It is therefore quite conceivable that many authors concentrate on determining these metabolites. In the past decade, we have witnessed a considerable increase in new analytical techniques dealing with the determination of sulphonamides. Among these procedures, especially the so-called multimethods using high-performance liquid chromatography —though sometimes including toilsome clean-up steps— can be mentioned. However, current approaches also utilize gas chromatography, gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, supercritical fluid chromatography–mass spectrometry, thin-layer chromatography and immunological methods. For most of these techniques, a strong trend towards lowering the level of detectability (down to the sub-ppb range) and improving accuracy and reproducibility can be established.

CONTENTS

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1. INTRODUCTION

Sulphonamides (SA) are a very important class of antibacterial compounds widely used in veterinary practice. Residues of these drugs and in many instances also their metabolites may occur in foods of animal origin and present a potential danger to consumers' health. Many of the parent compounds are approved for use in species under limitations which require withdrawal long enough to allow elimination of the residues from edible tissues.

This review deals with the analytical aspects of SA in meat. In Switzerland, as in many other countries, the legal tolerance is 0.1 mg/kg. This level includes the parent compounds and their possible N^4-acetylated metabolites. Many analytical methods have been applied to investigate concentrations of SA in meat. In the following, an attempt is made to summarize the different procedures developed since about 1982.

2. SAMPLE PREPARATION AND EXTRACTION PROCEDURES

The critical step in all SA residue methods is the clean-up, which is normally expected to remove constituents showing similar behaviour and/or reactions to the SA. Classical methodologies for the isolation of SA residues involve processes such as repeated homogenization of samples in an extraction or several extraction solvents, centrifugation of the extracts, back-extraction, pH adjustments, re-extraction and evaporation of the solvent. All these methods are more or less laborious and time-consuming and do not allow the analysis of large numbers of samples per day.

Long *et al.* [3] reported a new approach to the isolation of SA in catfish muscle tissue. With this procedure, called MSPD, the samples were blended into C_{18} by means of a glass pestle until the mixture appeared homogeneous. The semi-dry blend was transferred into a plastic syringe column and compressed. The column was then eluted with solvent(s) so as to isolate the SA. The authors proposed that the disruption of the tissue architecture that occurred was due both to mechanical shearing forces from blending and to hydrophobic forces of the covalently bound C_{18}. They stated that MSPD greatly speeded the process of SA residue screening.

As for the analytical methods, a considerable number of new techniques have been introduced to complement the already known methods in the last decade, such as EIA, ELISA, HPTLC, SFC and GC–tandem MS.

3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

3.1. Procedures without derivatization

In practice, SA screening for qualitative and quantitative residue control in meat is performed by RP-HPLC. The great advantage of HPLC is that SA may be analysed without derivatization and can be quantified by UV absorption at 254 or 270 nm. With more effort it is possible to analyse SA residues from meat with pre- or postcolumn derivatization to obtain results without matrix interferences. Some published methods are concerned with a single SA, others manage to extract the most important ones (up to twenty) from meat. Even multi-methods are known that permit the detection of up to 60 chemotherapeutics, antiparasites and growth promoters in one procedure [4].

Because of the inductive effect of the SO_2 group, SA are amphoteric (Fig. 1) and have different pK_a values, and consequently a work-up and clean-up procedure is aggravated. Several liquid–liquid and solid-phase sample clean-ups have been reported. Between pH 5.0 and 5.2 the commonly used SA are uncharged compounds [5].

In general, SA are poorly soluble in water, diethyl ether and chloroform, but readily soluble in polar organic solvents such as acetone. SA that are acetylated at the N^4-position, can be hydrolysed in boiling acidified acetone [5]. However, some workers have reported the determination of SA and their metabolites in one procedure without hydrolysation.

Usually, SA are fairly stable compounds, only a few being sensitive to UV radiation. Mainly, there is

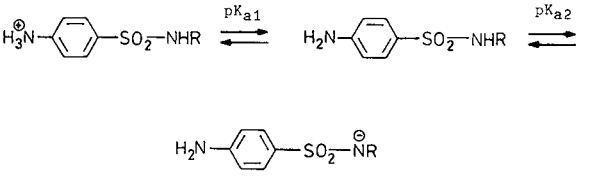

Fig. 1. Dissociation of sulphonamides.

an absorbance band at 270 nm, commonly used for UV detection, whereas fluorescence detection has been reported for SAA and SGU [6] and electrochemical detection is rarely used for SA [7].

Severe matrix interferences and low recoveries were the reason for developing highly sophisticated methods to determine different SA in one procedure. The isolation of SA residues from a complex mixture such as muscle, liver or kidney usually requires several time-consuming steps: homogenization of the sample with the extraction solvent; centrifugation; purification by solid-phase extraction or liquid–liquid partition by a series of washes, re-extractions and pH adjustments; and determination by RP-HPLC.

3.1.1. Simple routine methods to determine a few sulphonamides

Paulson and co-workers [8,9] reported the analysis of SMZ by HPLC in connection with pharmacological studies. Animal tissue was extracted with methanol, degreased with hexane, purified on an XAD-2 column and separated on a Radial-Pak C_{18} column.

Another study of SMZ and the N^4-metabolite in swine liver during frozen storage was described by Parks [10]. Meat was extracted with water, filtered and passed through a column of Duolite ES-863, a column of neutral alumina and a second column of Duolite ES-863 and chromatographed on Supelco LC-18.

Haagsma *et al.* [11] reported a method for the determination of SMZ in swine tissue. Extraction was performed with CH_2Cl_2 in an ultrasonic bath for 10 min. Clean-up was carried out by passing the extract through a Sep-Pak C_{18} cartridge. The purified extract was analysed on a Hypersil ODS column. Recovery for pig muscle tissue was reported to be 89.5%. Other determinations of SMZ were reported by Haagsma and co-workers [12,13].

A modified method by Haagsma and VanDeWater [14] can be used to determine five SA simultaneously (SAA, SMZ, SQX, SDA and SDO). After the extraction of meat with chloroform–acetone (1:1) and ultrasonic treatment, the acidified extract was passed through a 3-ml Baker cation-exchange column. A CP Spher C_8 column was used for HPLC analysis. Recoveries were reported to be high for muscle tissue (80–90%). However, low recoveries were sometimes found for kidney tissue, for unknown reasons.

Other pharmacokinetic studies have been published for SMZ, the N^4-acetyl metabolite and hydroxy metabolites in food-producing animals by Nouws and co-workers [15–19], but sample preparation and HPLC analysis were reported in only one of the papers [15].

Further pharmacokinetic studies have been described for SMZ in plasma, milk and uterine fluid of different animals [20,21], for SPR [22] and for SDA in the rat [23]. Good reviews on the pharmacokinetics of several differently used chemotherapeutics in animal husbandry were given by Lutz [24] and by Vree and Hekster [25].

Endoh *et al.* [26] reported the determination of SD in swine tissue, muscle, liver, kidney and fat. SD, widely used in Japan, was extracted with acetonitrile saturated with hexane. Further purification was achieved on an alumina phase. The purified drug was then analysed on a Nucleosil 5 C_{18} column. The reported recovery was 81–95%.

For SDM and OMP in tissue of cattle, chicken and catfish, a method was described by Weiss *et al.* [27]. Tissue was extracted at pH 10 with CH_2Cl_2 and tetrabutylammonium hydroxide, an ion-pair reagent, and both compounds were separated on a Porasil column. Tetrabutylammonium hydroxide is a reagent for ion-pair extractions, that is, anions from weak acids can be extracted with CH_2Cl_2 or $CHCl_3$ [28]. A similar strategy for the simultaneous extraction of STA and SMZ was used by Parks [29,30].

The same ion-pair extraction method was proposed by Rona *et al.* [31] for the determination of SPR in human serum. Further purification was obtained with an Extrelut column and on an RP-8 MOS-Hypersil column. They subsequently reported [32] a modified method for SPR in human serum and urine. The crude extract was purified with a Sep-Pak C_{18} cartridge. The analytical column used for separation was Hypersil ODS.

Petz [6] mentioned a liquid–liquid partition method for the determination of SAA and SGU. Muscle and liver tissue was extracted with acetonitrile at pH 8.5. Water was separated by adding sodium chloride, the evaporated residue was partitioned between aqueous methanol and hexane and the aqueous layer was used for chromatography at pH 2

on a Nucleosil 5 SA column. The native fluorescence was used for detection with excitation at 275 nm and emission at 340 nm.

Horii *et al.* [33] determined SMZ, SDM and SMM in animal tissue by extraction with acetonitrile and liquid–liquid partitioning. The residue was purified on a Bond Elut ODS cartridge at a relatively low pH and the three SA were separated on a Nucleosil 100 ODS column. The recovery in meat was 81–93%.

Another method for SMZ and its metabolites in body fluids was reported by Van 't Klooster *et al.* [34]. Samples were extracted and purified by liquid–liquid partitioning and separated on a Hypersil ODS column. This method can be applied to plasma, urine and cell culture media.

A new method for the determination of SDM and SMM in fish was published by Ueno *et al.* [35]. This method included the extraction of the two SA together with the N^4-acetylated metabolites with acetone and re-extraction with chloroform. The concentrated solution was cleaned up on a Sep-Pak alumina B cartridge and analysed on a YMC-Pack C_{18} column. An average recovery of 85% was measured at a level of 2 mg/kg.

3.1.2. Multi-methods to determine many sulphonamides

A simple and rapid method for thirteen SA residues (SAA, STA, SPR, SMZ, SCP, SDM, SGU, SAC, SDA, SME, SMP, SQX, SMX) in meat, liver and kidney, published by Rychener *et al.* [5], is also applicable to N^4-metabolites. A 10-g homogenized sample was extracted with acetone and partitioned between water and hexane. After neutralization, the SA were re-extracted with ethyl acetate and purified on a small silica gel column. The determination was effected by analysis on a Superspher 100 phase and on a cation-exchange phase (Nucleosil 5 SA) column. The SA were determined by UV detection at 270 nm. The two different HPLC analyses were necessary for confirmation purposes. It was not possible to separate all thirteen SA in one run without a gradient programme. The recovery was between 50 and 80%, depending on the type of SA.

Long and co-workers [36,37] published a simple multi-method for eight SA residues in milk which they called matrix solid-phase dispersion (MSPD). The same time-saving method was practicable with pork tissue [38] for SAA, STA, SDA, SME, SMZ, SMX, SDM and SIA. A 0.5-g sample of meat was mixed with 2 g of C_{18}-derivatized silica. The mixture was used to prepare a column. After washing the column with hexane, the SA were eluted with CH_2Cl_2. Without further purification the extract was chromatographed on an ODS column and no problems with interfering peaks were observed. Recoveries were between 70 and 95%.

Ikai *et al.* [39] reported a multi-method for ten SA residues in meat and fish (STA, SMX, SDA, SME, SMZ, SDM, SMP, SQX, SIZ and SMM). Ethyl acetate was used for extraction of 5 g of meat. Clean-up was performed with a Baker 10 amino cartridge. All ten SA were retained with ethyl acetate and eluted with 5 ml of acetonitrile. The separation was performed on Wakosil $5C_{18}$. The recoveries were 74–99%. This method is simple and rapid.

3.1.3. Multi-methods to determine many sulphonamides and other chemotherapeutics

For extensive residue monitoring control it is necessary to have multi-methods and to determine many different chemotherapeutics with one procedure. Single-residue determination is only efficient in case of suspicion.

Petz [40] proposed a multi-method for the determination of chloramphenicol, furazolidone and five SA (SDA, SME, SMZ, SMX and SQX) in meat, milk and eggs. A 25-g sample was extracted with acetonitrile, separated from co-extracted water and purified by liquid–liquid partitioning with hexane. For the analysis an MOS-Hypersil column was used and the detector was set at 275 nm. Recoveries were stated to be between 70 and 90%.

A new and extensive field was opened up by Malisch [4,41] with a multi-method for about 60 chemotherapeutics, antiparasitics and growth promoters in one procedure by combination of HPLC–UV detection and GC–ECD. After a complex and time-consuming sample preparation and clean-up, up to 28 SA were analysed on a Spherisorb ODS column with a gradient programme, a variable-wavelength detector and a photodiode-array detector. Recoveries for eleven SA were between 70 and 90%, but only 35% for SGU.

A method for the simultaneous determination of eleven synthetic antibacterial agents, including four SA (SME, SDM, SMM and SIZ), in cultured fish was described by Nose *et al.* [42]. A 10-g sample of

fish meat was extracted twice with acetone, purified by liquid–liquid partitioning and chromatographed on neutral alumina with different solvents. The drugs were analysed on a Nucleosil C_{18} column and with UV detection at 260 nm. Recoveries for the four SA were 70–91%.

Parks [7] described a screening procedure for six nitro-containing drugs, including one SA (SN), in chicken tissue. A 2.5-g amount of tissue was extracted with 20 ml of chloroform–DMSO–ethyl acetate (50:0.8:50) and purified on a small neutral alumina column. The drugs were eluted with phosphate buffer (pH 6)–methanol solution (1:1) and chromatographed on Supelcosil LC-18. Detection was effected with an amperometric detector with a glassy carbon electrode at −0.8 V *versus* Ag/AgCl. The recovery of SN was 91–97%.

A method for the simultaneous determination of eight antibacterial drugs, including three SA (SMZ, SMM and SIZ), used in cultured fish production was developed by Horie *et al.* [43]. The method is closely related to an earlier method to determine SMZ in meat [44]. The drugs were extracted with acidic methanol, followed by a Bond Elut clean-up procedure. Analysis of the drugs was carried out on an Intersil ODS column with detection at 265 nm. The recoveries for each drug added to the fish were 65–89%.

3.2. Procedures requiring derivatization

Fluorescamine {4-phenylspiro[furan-2-(3*H*),1′-(3′*H*)-isobenzofuran]-3,3′-dione; Fluram} and *p*-dimethylaminobenzaldehyde (DMBA) are widely used as derivatization reagents for SA in various TLC and HPTLC methods.

3.2.1. Precolumn derivatization

Takeda and Akiyama [45] derivatized specifically eight SA (SDA, SME, SMZ, SMX, SDM, SQX, SMM and SIM) with Fluram at pH 3 to give highly fluorescent compounds. The derivatized drugs were analysed on a Chemosorb 5-ODS-H column and detected with a fluorescence detector at an excitation wavelength of 405 nm and an emission wavelength of 495 nm.

3.2.2. Postcolumn derivatization

3.2.2.1. Derivatization with DMBA. SMZ and STA were easily extracted from feed and analysed using a LiChrosorb RP-18 column with detection at 450 nm after postcolumn derivatization with DMBA. This method was published by Smallidge *et al.* [46] as an extension of their earlier work [47].

A similar derivatization method and a combination of automated clean-up and concentration of milk, eggs and meat samples together with an HPLC separation was described by Aerts and co-workers [1,48]. Thirteen SA residues (STA, SQX, SAA, SMX, SME, SMZ, SGU, SDO, SDM, SDA, SAC, STR and SCP) and a few other drugs could be easily analysed in meat by automated on-line dialysis of the aqueous extract through a flat cellulose acetate membrane. The aqueous dialysate was concentrated on a small column (XAD-2 or XAD-4). After this concentration, the drugs were back-flushed with the HPLC eluent and analysed on a LiChrosorb RP-8 column. Because of many matrix interferences, a specific detection was necessary. For this reason, postcolumn derivatization with DMBA and sensitive detection of the SA residues at 450 nm were chosen.

3.2.2.2. Derivatization with Fluram. Sista *et al.* [49] determined SPR in human saliva by HPLC using postcolumn derivatization with Fluram and detection of the generated fluorophore with a fluorimetric detector at 395 nm (emission) and 470 nm (excitation). Fluram is expensive and only stable in a cooled solution for about 48 h. A similar method for the determination of twelve SA (SAA, SGU, SPR, SDA, SME, STA, SMZ, SMP, SCP, SDO, SDM and SMT) in meat and kidney was recently reported by Pacciarelli *et al.* [50]. The drugs were extracted with CH_2Cl_2–acetone (1:1). The extract was purified by solid-phase extraction on a cation-exchange cartridge (Chromabond SA 500) and chromatographed on LiChrospher 100 RP-18, followed by Fluram derivatization and fluorescence detection. Relative fluorescences and recoveries were reported.

4. GAS CHROMATOGRAPHY

GC methods for screening, quantification and confirmation could have the advantage of being more sensitive than LC. Sensitive detectors such as the electron-capture detector, in conjunction with

appropriate derivatizations, may improve the detectability, or a strong confirmation tool like MS could provide good identifications. The low vapour pressure and the high polarity of the SA necessitate derivatization. However, a few workers have described some technically advanced methods for the introduction of the underivatized SA into a mass spectrometer. Although these methods did not apply the GC technique, they are listed because of their mass spectrometric aspects.

Screening, confirmation and quantification of SDA, SDO, SMP, SMZ and SQX without derivatization were accomplished by Finlay *et al.* [51] in crude ethyl acetate extracts of pig kidney, introducing them by a solid probe or a moving belt interface into a hybrid tandem MS system and recording the collisionally activated (CAD) spectra. The mass spectrometer consisted of an electric and magnetic sector part, a quadrupole collision cell and a quadrupole mass analyser. Chemical ionization (CI) employing ammonia as the reagent gas and argon as the collision gas yielded daughter ion spectra suitable for confirmation by means of an intense protonated molecular ion and a set of fragments of the sulphanil moiety common to all the substances under study. Screening was accomplished by scanning the CI spectrum in the magnetic sector part and detecting the sulphanil fragment with the quadrupole mass analyser; detection limits of 100 μg/kg were achieved. A subsequent confirmatory experiment was performed by scanning the daughter ion spectra of the protonated molecular ions. The multiple ion detection mode was used for quantitative analysis by monitoring some intense daughter ions. The method was described as being very fast, and up to 400 crude extracts could be analysed before cleaning of the ion source became necessary.

Brumley *et al.* [52] applied collision-induced dissociation–mass analysed ion kinetic energy spectrometry (CID–MIKES) to the identification of SA in tissues. The CID–MIKE spectra of eighteen SA were presented. After a routine tissue clean-up, spiked liver samples were directly introduced with a solid probe and the spectra were obtained by isobutane chemical ionization and with helium as the collision gas. SDM, SMZ and SQX were determined in the range 100–200 μg/kg by scanning the electric sector in a magnetic sector instrument and recording the full-scan CID–MIKE spectra.

Manuel and Steller [53] reported a GC method for determining SBM, SCP, SDM, SMZ, SQX and STA in cattle and swine tissues. After an extraction by the Tishler method, the SA were methylated at the N^1-position by diazomethane, then separated on a packed column and detected with an electron-capture detector. The hydrolysis of the N^4-acetyl metabolite of SMZ in fortified tissue was studied. The methylation of SMZ by diazomethane was found to be approximately 90%. Recovery studies were performed in the range 100–1000 μg/kg.

Suhre *et al.* [54] developed an assay for SMZ using a packed column and determining the N^1-methyl derivative by MS with electron impact ionization. Quantification was accomplished after a modified Tishler clean-up and by comparing the ratio of two fragments of the derivative in the multiple-ion detection mode with the corresponding fragments of previously added ^{13}C-labelled SMZ. The method was tested on swine liver and muscle tissues, fortified with SMZ in the range 50–200 μg/kg.

In the investigation of incurred residues of SMZ, Matusik *et al.* [55] synthesized desaminosulphamethazine, N^4-acetylsulphamethazine, N^4-D-glucosylsulphamethazine and N^4-(1-deoxy-D-glucuronyl)sulphamethazine. In order to compare the Tishler spectrophotometric method and the GC methods of Manuel and Steller [53] and Suhre *et al.* [54], swine liver and muscle tissues were fortified with SMZ and the metabolites mentioned above in the range 100–200 μg/kg. The N^4-derived metabolites yielded poor chromatographic and recovery properties. With the addition of a hydrolysis step using dilute hydrochloric acid, all metabolites were determined as N^1-methylsulphamethazine or N^1-methyldesaminosulphamethazine, respectively, by GC. Recovery studies were reported for the different metabolites and assays.

Paulson *et al.* [56] described another procedure for the identification and determination of ^{14}C-labelled SMZ, N^4-acetylsulphamethazine, N^4-glycosylsulphamethazine and desaminosulphamethazine in swine tissue after oral administration of laballed SMZ. After clean-up of the tissue, the substances in question were separated by RP-LC and determined by measuring the carbon-14 activity by liquid scintillation counting. The fractions of these substances were collected separately and methylated with di-

azomethane; N^4-glycosylsulphamethazine had to be hydrolysed before derivatization and GC. The derivatives were subsequently analysed by on-column injection capillary GC and full-scan MS with electron-impact ionization. Determinations were carried out routinely in the range 50–100 μg/kg, but also a few μg/kg could be detected.

Stout *et al.* [57] developed a confirmatory method for SMZ in cattle and swine tissue. After a clean-up, a derivatization step in accordance with Manuel and Steller [53] provided that N^1-methylsulphamethazine, which was determined by packed-column GC and by CI-MS, using methane as the reagent gas. The advantage of the CI mode was that the spectrum generated an intense protonated molecular ion and a fragment indicative of the methylated amine functionality of the molecule in the positive-ion CI mode and an intense fragment of the sulphanil moiety in the negative-ion CI mode. In this way and with an accessory for pulsed positive-ion–negative-ion CI, it was possible to detect a few nanograms of SMZ. Satisfactory recoveries were obtained in cattle and swine tissues in the 100 μg/kg range, and even residues of less than 10 μg/kg were confirmed.

Matusik *et al.* [58] modified the method of Manuel and Steller [53] using GC–ECD in order to separate and determine SMZ and two of its metabolites, N^4-acetylsulphamethazine and desaminosulphamethazine, and applied it to incurred residues in cattle and swine tissues. This method, performed on a packed column, provided the contents of tissues of dosed animals after a 1-week withdrawal time. These derivatives were also separated on a short, non-polar capillary column and identified by positive-ion CI-MS, using methane as reagent gas. Full-scan spectra were provided and residues of fortified and incurred tissues were confirmed in the multiple-ion detection mode.

In a later approach, Matusik *et al.* [59] extended the method by ECD to SMZ, SDM, SCP and STA, all of which have the highest violation rate in the USA, and to a confirmation method using tandem MS. As in the procedure mentioned above, the extraction was accomplished by a modified Manuel–Steller or Tishler method and quantification was performed with ECD of the N^1-methylated substances. Recovery studies on fortified and incurred cattle and swine tissues were presented. The confirmation method by quadrupole tandem MS was performed by using a short capillary column for the separation and GC introduction of the N^1-methylated SA, ammonia as the reagent gas for positive-ion CI (this providing the most abundant protonated molecular ions) and argon as the collision gas for the daughter ion experiments. The daughter ion spectra derived from the protonated molecular ions contained the latter and ions corresponding to the sulphanil moiety, the methylated amino moiety and cleavage products of the sulphanil part. The procedure had sufficient sensitivity to provide full-scan daughter ions spectra in tissue residues at the 100 μg/kg level. This method provides better information than the multiple-ion detection mode.

Takatsuki and Kikuchi [60] described a method based on the N^1-methylated derivatives for SMZ, SDM, SME, SMX and SQX using capillary GC and MS with EI ionization in the multiple-ion detection mode. They focused on faster eluting by-products with similar fragmentation modes but mass spectra different from those of het isomers reported by Feil *et al.* [61]. The formation of these by-products seemed to be favoured during methylation by diazomethane by light or heating to evaporation.

Carignan and Carrier [62] desorbed a determination and confirmation procedure based on a clean-up by extraction and LC and subsequent N^1-methylation, followed by determination by GC and MS with EI ionization in the multiple-ion detection mode. SA in fortified swine tissues were measured in the range 1–100 μg/kg.

The formation of isomeric by-products in the N^1-methylation step of derivatizations was first studied by Gilbert *et al.* [63]. An isomer of N^1-methylsulphapyridine was isolated and characterized by ^{1}H NMR and MS. It was shown that to some extent the methylation took place at the pyridine-nitrogen. Similar effects could be observed with SMZ and SDA, the greatest amount of the by-product occurring with SPR at levels of a few percent. A similar by-product was obtained in a large-scale methylation of SMZ by Feil *et al.* [61] and was characterized by means of ^{1}H NMR spectroscopy and fast atom bombardment (FAB) MS as a tautomeric methylation product, the methylation taking place at a pyrimidine-nitrogen. Fortified tissues showed different yields of the by-product in the range 8–30%.

Although N^1-methyl derivatives of SA provided

good GC properties and the possibility of applying sensitive detection techniques such as ECD or MS in different ionization modes, several attempts were made to improve these properties by acylating the sulphanil amino group.

Gyllenhaal *et al.* [64] described the extractive alkylation with pentafluorobenzyl halides and a subsequent acylation with heptafluorobutyric or trifluoroacetic anhydride in order to detect the derivatized SA by ECD.

Garland and Miwa [65] proposed a method to determine SDM in cattle and swine. After clean-up, the extract was treated with diazomethane and pentafluoropropionic anhydride. The derivative was detected at the residue level by positive-ion CI using isobutane as the reagent gas by multiple ion detection. For quantification, deuterated SDM was used, the synthesis of which was described. The spectra showed a protonated molecular ion and a fragment corresponding to the amine part. Data for the spectra of derivatized SMZ, SCP, SDA, SDM, SDO, SMP, SPY, SQX and STA were listed. The method was tested on spiked tissue samples in the range 50–200 μg/kg. The identification of SDM or SDO was difficult because of the almost identical mass spectra of these isomeric substances and of the restricted resolution of packed-column chromatography.

Roach *et al.* [66] listed the EI and positive- and negative-ion CI mass spectra, using methane as reagent gas, of seventeen SA and of the N^1-methyl- and N^1-methyl-N^4-pentafluoropropionyl derivatives of SMZ. Extensive interpretations were given for these spectra. Positive-ion CI yields intense fragments such as the protonated molecular ion and the amine part of the substance or the derivatives, respectively. Negative-ion CI showed an intense ion corresponding to the sulphanil moiety. The authors suggested applying these fragmentation properties in a pulsed positive-ion–negative-ion CI procedure for detection at the residue level.

The good GC properties of the N^1-methyl-N^4-heptafluorobutyryl derivatives were described by Holtmannspötter and Thier [67]. Capillary GC was used in conjunction with flame ionization detection. The clean-up of the tissue extracts involved a gel chromatographic step and recovery data for SAA, SDA, SME, SMZ, STA and SQX were listed at concentrations of 100 μg/kg in tissue, eggs and milk, together with data for chloramphenicol and furazolidone. The detection limit was 10 μg/kg.

Simpson *et al.* [68] determined SMZ, SBM, SDM, SQX and STA after an extraction according to Tishler. The substances were methylated with diazomethane and acylated with pentafluoropropionic anhydride. The method was elaborated with fortified tissues using packed-column GC and MS with EI ionization in the single-ion monitoring mode. Quantification was performed by using the ^{13}C-labelled substances as internal standards. Recovery studies were presented in the range 50–200 μg/kg.

Mooser and Koch [69] proposed a confirmation method following quantification by LC. SMZ, SCP, SDA, SDM, SME, SMP, SMX, SPR, SQX and STA were determined as the N^1-methyl-N^4-trifluoroacetyl derivatives by capillary GC–MS. Extracts were methylated with diazomethane and trifluoroacetylated with N-methylbistrifluoroacetamide. The positive-ion CI mass spectra using methane as the reagent gas yielded intense ions corresponding to the protonated molecular ion and to the amine moiety of the derivatives, and in the negative-ion mode the sulphanil part yielded very intense ions. Prior to the derivatization, SGU was cyclized with hexafluoroacetylacetone to give the fluorinated analogue of SMZ. SAC was methylated with iodomethane and showed similar fragmentations. Detection was performed at the residue level using pulsed positive-ion–negative-ion CI. SAA was detected as the methyl derivative by EI ionization in the multiple-ion detection mode. The method was routinely tested on violation samples of cattle, swine and rabbit.

Kmostak and Dvorak [70] described a capillary GC-ECD method in order to determine SDM with an external standard. The clean-up involved a sorption step with extractive alkylation using iodomethane and subsequent acylation with trifluoroacetic anhydride. The method was applied to incurred residues in swine tissue with a detection limit of 10 μg/kg.

5. COUPLED TECHNIQUES

5.1. Liquid chromatography–mass spectrometry

Henion *et al.* [71] applied atmospheric-pressure CI in conjunction with LC to the MS determination

of SMZ, SDA and SDM in racehorse urine. The mass spectra were very simple and the authors described a tandem MS system producing more characteristic daughter ion spectra.

Horie *et al.* [72] described the detection of SMZ, SCP, SDA, SDO, SDM, SME, SMX, SQX and STA in meat by means of LC and thermospray MS. The mass spectra yielded mainly the protonated molecular ion. These ions were used in the single-ion monitoring mode for determination by means of external standards. Chromatographic analyses of incurred swine tissue at the level of 1 mg/kg were shown.

Pleasance *et al.* [73] reported the separation and identification of 21 SA by RP-LC and ion-spray MS. In accordance with the results of Henion *et al.* [71], positive-ion mass spectra yielded only abundant protonated molecular ions. Further information was provided by tandem MS, giving more structural information by daughter ion spectra. Detection of SDM in incurred salmon tissue at the level of 25 μg/kg using a diode-array UV spectrometer as compared with LC–MS by single-ion monitoring using the protonated molecular ion for confirmation.

5.2. Supercritical fluid chromatography–mass spectrometry

Perkins *et al.* [74] reported a packed-column SFC separation on silica or amino-bonded silica, using carbon dioxide with methanol modifier as the mobile phase. The effects of column pressure and modifier concentration were studied in the separation of SMZ, SCP, SDA, SDO, SME, SMP, SPY, SQX and STA. Detection was accomplished by UV spectrophotometry or MS using moving-belt or thermospray interfaces. EI and ammonia CI mass spectra were presented and showed simple spectra, mainly with the protonated molecular ion as the base peak. The possibility of detecting residues in spiked tissue was tested on SMZ at the level of 3 mg/kg, comparing UV absorption with MS by single-ion monitoring and a moving-belt interface.

6. THIN-LAYER CHROMATOGRAPHY

LC and GC are capable of detecting SA at the low-μg/kg level. TLC was described by Horwitz [75] as lacking sensitivity and precision for quantification. Meanwhile, several approaches have been published (see Table 1) in order to detect or even determine SA in nanogram amounts in edible tissues by means of TLC. In fact, the technique has several distinct advantages over other chromatographic methods: the possibility of analysing many samples simultaneously rather than serially and, in conjunction with newer HPTLC materials giving shorter run times, results in significant time savings. The use of selective detection reagents such as the Bratton–Marshall reagent or fluoresamine can provide sensitive and, with a scanner, rapid quantitative assays. Bratton *et al.* [86] introduced N-(1-naphthyl)ethylenediamine as a diazotization reagent, whose application was extensively discussed by Horwitz [75]. Parks [80] proposed a modified version for the rapid development of thin-layer plates. Fluorescamine (Fluram) reacts very rapidly with primary amines, forming intensely fluorescent derivatives.

7. NON-CHROMATOGRAPHIC METHODS

Dixon-Holland and Katz [87] described a direct competitive ELISA for the detection of SMZ in swine urine and muscle tissue. Urine without any clean-up or an extract of muscle were analysed by absorbance measurement, using 2,2′-azino(3-ethylbenzothiazoline)sulphonic acid as a chromogen. SMZ was detected in concentrations as low as 20 μg/kg in muscle and 10 μg/kg in urine.

A similar competitive solid-phase EIA for the detection of SMZ in swine plasma was developed by Singh *et al.* [88] for the concentration range 10–1000 μg/kg. Validation values and comparison with TLC were reported for plasma, obtained without any clean-up step. Among the 36 SA studied, only SME showed a cross-reaction in the assay.

Ram *et al.* [89] presented an EIA in order to determine SMZ in swine plasma or serum with larger handling volumes of sample solutions and an automated technique. Comparison with TLC showed a good correlation in the range 1–5 μg/kg. The assay was tested on SMZ-fed pigs and their plasma and serum.

8. CONCLUSIONS

There has been an enormous increase in new analytical procedures on the one hand and consider-

TABLE I

SUMMARY OF TLC METHODS

Ref.	Sulphonamides	Purpose	Type of plate	Type of extraction	Type of detection	Detection limits, remarks
76	SMZ, SAA, SCP, SDA, SDM, SDO, SGU, SME, SMP, SMX, SPR, SQX, STZ	TLC:screening HPLC:quantification, except for SGU, SAA	HPTLC, silica gel, 3 eluents HPLC:RP-18, acetate buffer (pH 4.6)–methanol	10 g tissue, solid–liquid extraction, CH_2Cl_2	HPTLC:fluorescamine HPLC:UV (266 nm)	HPTLC:20 μg/kg HPLC:5 μg/kg
77	SAA, SDA, SMZ, SQX, SDO	Screening	Silica gel, $CHCl_3$–C_4H_9OH (4:1)	10 g tissue, solid–liquid extraction, CH_2Cl_2	Fluorescamine	50 μg/kg
78	SAA, SDA, SMZ, SQX, SDO	Screening	Silica gel, $CHCl_3$–C_4H_9OH (4:1)	10 g tissue, solid–liquid extraction, CH_2Cl_2	Fluorescamine	50 μg/kg Collaborative study
79	20 SA	Screening	HPTLC, silica gel, 4 eluents	5 g tissue, solvent extraction, C_2H_5OAc	Fluorescamine	100 μg/kg
80	SMZ, STZ	Screening	Silica gel, EtOAc–CH_3OH (4:1)	2.5 g tissue, solvent extraction, $CHCl_3$–C_2H_5OAc	Bratton–Marshall	20 μg/kg
81	SDM, SQX	Screening	Silica gel, $CHCl_3$–EtOAc–CH_3OH (5:5:1)	5 g tissue, solvent extraction, $CHCl_3$–C_2H_5OAc (1:1)	Bratton–Marshall	< 100 μg/kg Tested: 100–400 μg/kg Also coccidiostaticss and their metabolites
82	23 SA	Screening, quantification	HPTLC, silica gel, 6 eluents, two-dimensional	2 g tissue, solvent extraction, (1) buffer, acetone–CH_3OH or (2) C_4H_9OAc	Fluorescamine	50 μg/kg (serum: 5 μg/kg)
83 84	SMZ, SDM, SQX	Screening, quantification	Silica gel, CH_3OH, $CHCl_3$–C_4H_9OH (4:1)	2.5 g tissue, solvent extraction, C_2H_5OAc	Fluorescamine, densitometry	Tested: 50–200 μg/kg Collaborative study (comparison with Tishler and Manuel–Steller methods)
85	16 SA	Screening	HPTLC, silica gel, 3 eluents, two-dimensional	10 g tissue, solid–liquid extraction, $CHCl_3$–acetone (1:1)	Fluorescamine	10 μg/kg (10 ng)

able progress in lowering detection limits of SA on the other in the past decade. There is no doubt that reducing the level of detectability is still continuing despite the fact that many methods reach the sub-ng/g range. In this context the question might arise of whether this trend is reasonable from a practical point of view. It can be stated that a great many methods for SA in meat and meat products exist and that there is, although perhaps sounding rather presumptuous, no or little actual need for additional ones.

Various workers are paying great attention to problems arising from clean-up and possible metabolites of originally administered SA. A new and promising approach seems to be the matrix solid-phase dispersion isolation of substances, although the limits of this procedure are not yet clear.

9. ABBREVIATIONS

The pK_a values (SO_2-NH) are taken from refs. 1 and 2.

DMSO	Dimethyl sulphoxide
ECD	Electron-capture detection
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
MS	Mass spectrometry
MSPD	Matrix solid-phase dispersion
RP	Reversed-phase
SFC	Supercritical fluid chromatography
TLC	Thin-layer chromatography
DMBA	*p*-Dimethylaminobenzaldehyde
SA	Sulphonamide(s)
OMP	Ormetoprim
SAA	Sulphanilamide (Sigma S-9251; pK_a 10.43)
SAC	Sulphacetamide (Serva 35630; pK_a 5.38)
SBM	Sulphabrommethazine
SCL	Sulphaclozine
SCP	Sulphachlorpyridazine (Sigma S-9892; pK_a 5.1)
SD	Sulphamoyldapsone
SDA	Sulphadiazine = sulphapyrimidine (Sigma S-8626; pK_a 6.4)
SDM	Sulphadimethoxine (Sigma S-7007; pK_a 6.2)
SDO	Sulphadoxine
SFU	Sulphafurazole
SGU	Sulphaguanidine (Sigma S-8751; pK_a 11.25)
SIA	Sulphisoxazole
SIM	Sulphisomidine
SIZ	Sulphisozole
SME	Sulphamerazine (Sigma S-8876; pK_a 7.0)
SMM	Sulphamonomethoxine
SMP	Sulphamethoxypyridazine (Sigma S-7257; pK_a 6.7)
SMT	Sulphamethizole
SMX	Sulphamethoxazole (Sigma S-7507; pK_a 5.6)
SMZ	Sulphamethazine = sulphadimidine (Serva 35635; pK_a 7.4)
SN	Sulphanitran
SPE	Sulphaperine
SPH	Sulphaphenazole
SPR	Sulphapyridine (Serva 35860)
SPY	Sulphapyrazole
SQX	Sulphaquinoxaline (Sigma S-7382; pK_a 5.5)
STA	Sulphathiazole (Serva 35690; pK_a 7.2)
STO	Sulphatolumide
STR	Sulphatroxazole (pK_a 5.8)

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Review

Phycotoxins in seafood —toxicological and chromatographic aspects

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ABSTRACT

Two typical clinical types of algae-related seafood poisoning have attracted medical and scientific attention: paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP). Therefore, it became necessary to establish methods for the evaluation of possible hazards caused by contamination of seafood with these phycotoxins. Bioassays with mice or rats are the common methods for the determination of the toxin content of seafood. However, biological tests are not completely satisfactory because of a lack of sensitivity and pronounced variations. Additionally, there is growing opposition against animal testing. Therefore, many efforts have been undertaken to determine phycotoxins by chromatographic methods. PSP determination is mainly based on high-performance liquid chromatographic (HPLC) separation by ion-pair chromatography followed by postcolumn oxidation of the underivatized toxins in alkaline solution and fluorescence detection. HPLC methods for the determination of the DSP toxins okadaic acid (OA) and dinophysistoxin-1 (DTX-1) are characterized by precolumn derivatization with 9-anthryldiazomethane (ADAM) and/or 4-bromomethyl-7-methoxycoumarin (Br-Mmc), followed by chromatographic separation of the DSP esters formed and fluorescence detection. The chromatographic methods discussed in this review allow the rapid, sensitive and non-ambiguous determination of individual species of the two most important phycotoxins in seafood, PSP and DSP.

CONTENTS

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1. INTRODUCTION

It has been known since ancient times that certain fish and shellfish may be poisonous and can cause death after consumpion [1]. The chemical nature and biological basis for these food-borne intoxications have been elucidated during the last 50 years. It is now evident that certain microscopic algae produce very potent toxins (phycotoxins or algal toxins). The concentrations of toxins in the sea are highest during algal blooms. The phycotoxins are taken up by predators feeding on plankton, either directly as with molluscs or through several trophic levels as in fish. These food items are then consumed by man (Fig. 1).

As fish and shellfish constitute an important part of the world's food supplies, the apparently increasing contamination of food by algal toxins constitutes a specific chemical hazard requiring appropriate attention [3,4].

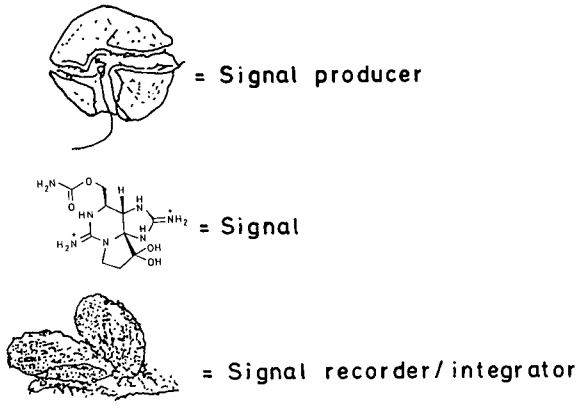


Fig. 1. The common mussel, found world-wide, is used as the signal recorder/integrator [2].

1.1. Paralytic shellfish poisoning (PSP)

1.1.1. Occurrence in seafood

The PSP toxins are produced by certain unicellular marine algae known as dinoflagellates. Most of the PSP-producing dinoflagellates are found in the genus *Alexandrium* [5]. Contamination of shellfish with PSP toxins has traditionally been associated with the appearance of an algal bloom, the so-called "red tide". It is also important to note, however, that not all red tides are associated with toxic phycoplankton and contamination of shellfish. On the other hand, shellfish can still contain PSP when *Alexandrium* spp. concentrations in the sea are already far below those found in algal blooms.

The PSP compounds are transferred to shellfish (*e.g.*, mussels, clams, scallops) during filter-feeding. Digestion takes place in the intestinal tract and is associated with the hepatopancreas complex. The highest concentrations of PSP have been found in these digestive glands [4].

1.1.2. Chemical structure and toxicity

PSP symptoms include perioral tingling sensations, prickly sensations in the finger tips and toes, progressing to arms and legs, then general weakness and slight respiratory insufficiency, followed by muscular paralysis, severe respiratory difficulty and finally death unless ventilatory support is given [6].

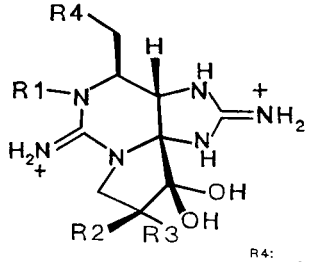
The first PSP component to be chemically characterized was saxitoxin (STX), which, although it was initially discovered in shellfish in California, has since been found in the greatest concentrations in the Alaska butterclam, *Saxidomus giganteus*, from which the name was derived [7]. Saxitoxin is a hygroscopic solid, soluble in water and methanol, but almost insoluble in most non-polar organic solvents [8].

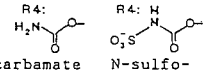
Subsequently, several other toxins of the PSP group have been characterized chemically, including 1-hydroxysaxitoxin (NEO) and the epimers of 11-hydroxysaxitoxin sulphate and 11-hydroxyneosaxitoxin sulphate [9–12]. The last compounds, named gonyautoxin II, III, I and IV (GTX), are less basic, but otherwise their properties are similar to those of STX.

In addition, PSP compounds with a sulphocarbamoyl group have been isolated from both dinoflagellates and shellfish, and decarbamoyl toxins, *e.g.*, decarbamoylsaxitoxin (dc-STX), which previously had been made only in the laboratory, has been found in nature [13–17].

Thus, referring to their chemical structure, three groups of PSP toxins (N-sulphocarbamoyl, carbamate and decarbamoyl toxins) are known (Fig. 2).

The nature and number of PSP compounds depend on the toxin patterns produced by the dinoflagellates, their storage and their metabolism in shellfish [18,19]. In addition to the differences in chemical structure, the individual PSP toxins show various toxicity. Originally, toxicities were expressed in mouse units (MU, or the amount of toxin that kills a 20-g mouse in 15 min), but with the introduction of pure saxitoxin as a standard substance for toxicity control this was refined to 1 microgram saxitoxin equivalents, usually being referred to 100 g of shellfish meat [14]. Th absolute toxicity of STX was assigned to be 5500 MU/mg,





R1	R2	R3	carbamate toxins	N-sulfo-carbamoyl-toxins	decarbamoyl-toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO_3^-	GTX I	C3	dc-GTX I
H	H	OSO_3^-	GTX II	C1	dc-GTX II
H	OSO_3^-	H	GTX III	C2	dc-GTX III
OH	OSO_3^-	H	GTX IV	C4	dc-GTX IV

Fig. 2. Structure of naturally occurring PSP components [16]. STX = saxitoxin; NEO = neosaxitoxin; GTX = gonyautoxins.

where 1 MU is equivalent to 0.18 μg of STX as the dihydrochloride [11]. The value of 1.0 was established for the relative toxicity of STX [14].

Table 1 gives the specific toxicities of individual PSP toxins (absolute toxicity, expressed in MU/μ*M*). The toxicity of the N-sulphocarbamoyl toxins is relatively low, that of the carbamate toxins is significantly higher and the decarbamoyl toxins exhibit intermediate toxicity [20].

In general, PSP toxins are heat stable at slightly acidic pH, but unstable and easily oxidized under alkaline conditions [4]. It is possible that PSP toxins are converted by enzymatic processes: the hydrolysis of N-sulphocarbamoyl toxins in clams leads to the more toxic decarbamoyl toxins. At the end of the metabolic process often only dc-STX is present [21]. One may assume that in man gastric hydrochloric acid also converts N-sulphocarbamoyl toxins into carbamate and decarbamoyl toxins, thus increasing the toxicity of PSP-contaminated seafood [16].

1.2. Diarrhetic shellfish poisoning (DSP)

1.2.1. Occurrence in seafood

After the consumption of shellfish which have been feeding on toxic dinoflagellates of the *Dinophysis* or *Prorocentrum* genera, intestinal disturbances such as diarrhoea, nausea, vomiting, abdominal pain and chills may occur. As in most cases diarrhoea is the predominant symptom, the syndrome is called diarrhetic shellfish poisoning (DSP) [22,23]. Species of DSP-producing algae are widely distributed but seldom form red tides. *Dinophysis fortii* has been identified as a producer of DSP in Japan [24], whereas *Dinophysis acuminata* is suspected to be the toxin producer in recent outbreaks in Netherlands and Germany [25–27]. It has been reported that in the presence of *Dinophysis fortii* at a low cell density of 200 cells/l, mussels and scallops became toxic enough to affect man [27].

1.2.2. Chemical structure and toxicity

The heat-stable, lipophilic substances of the DSP complex are classified into three groups: okadaic acid (OA) and its derivatives named dinophysistoxins (DTX), the pectenotoxins (PTX) and yessotoxin (YTX) [23] (Fig. 3).

OA and DTX were obtained from the digestive

TABLE 1

SPECIFIC TOXICITY OF PSP TOXINS [20]

N-Sulphocarbamoyl toxins		Carbamate toxins		Decarbamoyl toxins	
Toxin	Specific toxicity (MU/μM)	Toxin	Specific toxicity (MU/μM)	Toxin	Specific toxicity (MU/μM)
B1	250	STX	2100	dc-STX	900
B2	250	NEO	2300	dc-NEO	900
C1	17	GTX2	1000	dc-GTX2	380
C2	258	GTX3	1600	dc-GTX3	380
		GTX1	1900	dc-GTX1	950
		GTX4	1900	dc-GTX4	950

glands (hepatopancreas) of the blue mussel *Mytilus edulis* and some of the toxic compounds PTX and YTX have been identified in the digestive glands of the scallops *Patinopecten yessoensis* [28–31]. Among these toxins, only OA, DTX-1 and DTX-3 have been proved to induce diarrhoea in man [32]. Further measurements revealed that OA is the principal toxin in European mussels, whereas DTX-1 predominates in Japanese scallops [33,34]. Therefore, the monitoring of seafood for DSP toxins with high-performance liquid chromatographic (HPLC) methods is restricted to OA and DTX-1 determination [35].

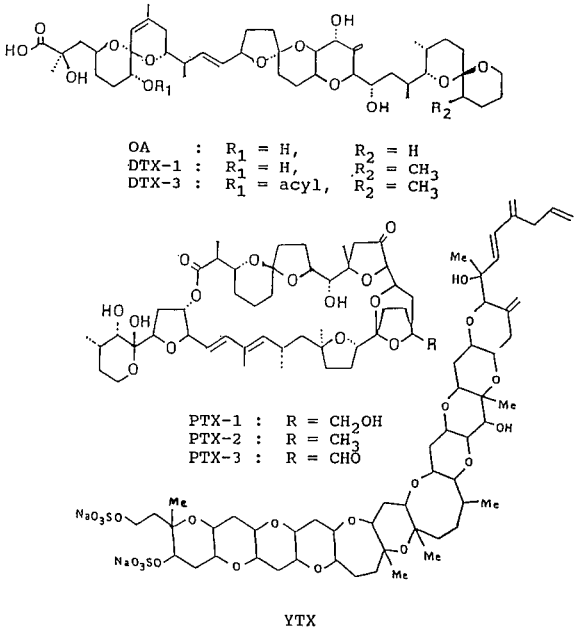


Fig. 3. Structure of DSP components [28]. OA = okadaic acid; DTX = dinophysistoxin; PTX = pectenotoxin; YTX = yessotoxin.

1.3. Regulations for phycotoxins in seafood

Marine phycotoxin regulations may involve environmental surveillance for toxic algal species in areas where shellfish are grown and legal actions to ensure that toxin-contaminated shellfish do not reach the consumer. Therefore, several countries have monitoring programmes to check for the occurrence of toxic phytoplankton [28].

Weighing the various factors that play a role in the decision-making process of establishing phytotoxin tolerances may not be easy. Despite this dilemma, a number of countries have established limits and regulations for marine phycotoxins [36]. Regulations exist for PSP and specifically for saxitoxin, and for DSP and specifically for okadaic acid [37].

Actual or proposed tolerance levels for PSP are typically 400 MU per 100 g; 40–80 μg PSP per 100 g and 40–80 μg saxitoxin per 100 g. For DSP the tolerance levels also vary: 5 MU per 100 g, not detectable in rat bioassay, and 20–60 μg DSP per 100 g (Table 2).

Different concentration limits are used to express the tolerance level for PSP: MU per 100 g and μg per 100 g. The latter unit currently seems to be less appropriate in the countries that use the mouse bioassay, because they actually test for toxicity only in the mouse [37]. Expression of a tolerance level for PSP in μg per 100 g would be valuable if the various PSP exhibit the same toxicity, but they do not [38].

TABLE 2

SUMMARY OF REGULATIONS FOR PSP AND DSP [37]

	PSP	DSP
Products	Molluscs, shellfish, bivalves, mussels	Molluscs, shellfish, bivalves, mussels
Limits	400 MU per 100 g 40–80 μg PSP per 100 g 40–80 μg saxitoxin per 100 g	5 MU per 100 g 20–60 μg per 100 g Not detectable in a rat bioassay
Methods	Mouse bioassay Spectrometry HPLC	Mouse bioassay Rat bioassay HPLC

To overcome the differences between the findings of the mouse bioassay and the results of PSP determinations by HPLC, accurate HPLC methods permitting the exact determination of all individual PSP components in seafood samples must be available [39–41]. The tolerance levels for DSP were generally set at the limit of detection of the analytical method used, most often a mouse bioassay; a few countries relied on a rat bioassay and incidentally HPLC in addition.

The most modern approach for the determination of both PSP and DSP is HPLC [37]. The application of HPLC for regulatory purposes, however, is hampered by the lack of validated analytical methodology, pure analytical standards of the various toxins and reference samples for analytical purpose. Neither the bioassays nor the HPLC procedures have been validated in collaborative studies. Therefore, developments in this area deserve strong support, because the enforcement of phycotoxin regulations ultimately depends on reliable analytical measurements [42].

2. ANALYTICAL METHODOLOGY

2.1. Methods for PSP determination

2.1.1. Bioassay

The highly potent and unpredictable nature of PSP necessitates constant monitoring of the toxin content of shellfish from beaches in affected areas. These monitoring programmes use the standard mouse bioassay for PSP determination as prescribed by the AOAC for determining the level of toxicity [43]. It consists of the intraperitoneal (i.p.) injection into mice of an acidified and heated extract of shellfish tissues and the determination of the time until death occurs [44]. By standardizing the conditions for the bioassay (mouse mass, pH of extract and salt concentration) a fairly reliable routine procedure was established [4]. Because different strains of mice differ in their susceptibility to the PSP toxins, the sensitivity of the mouse colony used must first be determined by calculating a correction factor (CF value) obtained after i.p. injection of the STX standard. The acidified extracts of shellfish are screen-tested in a few mice in order to determine the dilution of the extract that will kill mice of 19–21 g body mass within 5–7 min, the conditions under which the assay is most sensitive. PSP levels of samples as low as about 400 μg STX equivalent per kilogram of seafood can be detected [45].

The requirement for a large number and constant supply of mice is often cited as the main drawback of this bioassay [46]. An additional problem is the protective effect of NaCl; Schantz *et al.* [44] studied the effect of salt on the death times of mice and found that, at the 0.5% NaCl level, a 30% lower value was obtained for PSP toxin concentration. Further, the bioassay detects lethal toxicity in a sample, regardless of the chemical structure of the toxins. This may have advantages from a regulary standpoint but is a disadvantage when individual PSP toxins are to be determined. In view of these facts, chromatographic techniques have been developed to separate individual PSP toxins [47,48].

2.1.2. HPLC for PSP determination

HPLC techniques allow the separation and sensitive detection of individual PSP toxins irrespective of their number and group. Therefore, HPLC methods have opened up a new dimension in phycotoxin analysis [14]. However, the results obtained have to be comparable to those of the mouse bioassay [40]. This requirement is partly fulfilled by the application of identical procedures for sample preparation (Fig. 4).

Additionally, accurate HPLC determination of the various PSP components in the samples is a necessity.

The concentrations of individual PSP toxins were calculated on the basis of the PSP peaks in the HPLC traces, converted into their STX equivalent

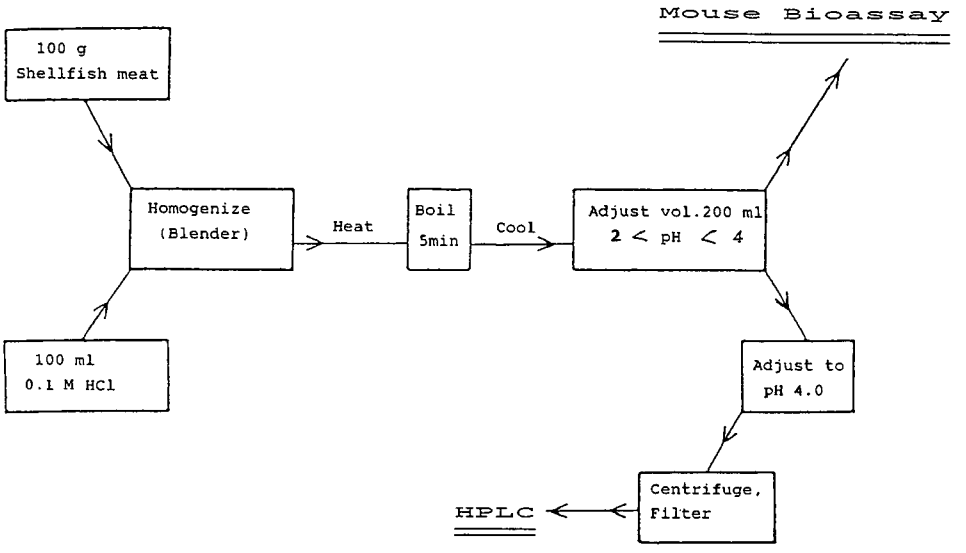


Fig. 4. Sample preparation procedure for comparative studies involving HPLC and mouse bioassay [39].

and summed for comparison with bioassay values [39]. Individual contributions to sample toxicity (G) were calculated for each toxin using the following equation [40]:

$$G = CTD/100$$

where C = toxin concentration (μm per 100 g), T = toxicity factor (μg STX/μM toxin) and D = dilution of sample (ml per 100 g of shellfish meat).

2.1.2.1. Toxin detection

The detection of the PSP toxins is based on the fluorimetric assay described by Bates and co-workers [50,51]. As PSP toxins show neither UV absorption nor fluorescence, STX was oxidized in alkaline solution to obtain derivatives detectable by common HPLC detectors. The derivatization reaction is based on the oxidation of STX to 8-amino-6-hydroxymethyl-2-aminopurine-3-propionic acid, which reacts in acidic solution to give a fluorescent pyrimidopurine (Fig. 5).

For PSP detection some workers have used this reaction and measured directly the fluorescence of the oxidation products [52,53]; others first separated the oxidation products by chromatography before subsequent fluorescence detection [54,55]. It should be noted that the application of the oxidation reaction for precolumn derivatization has several drawbacks. For example, our experiments with STX revealed that following oxidation with $NaOH–H_2O_2$ more than one peak appeared in the HPLC traces. In addition, the analysis of canned mussels contaminated with PSP revealed the presence of large amounts of decarbamoyl toxins in addition to carbamate toxins. However, up to now no chromatograms have been published showing the chromatographic separation of defined oxidation products of decarbamoyl toxins after precolumn derivatization [56,57]. Therefore, the oxidation of PSP toxins is usually carried out as a postcolumn reaction [58]. When this technique is applied the number of oxidation products of individual PSP

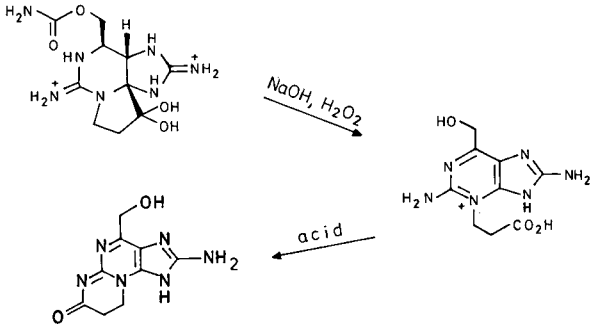


Fig. 5. Oxidation of saxitoxin to fluorescent pyrimidopurine [50].

components is not important, because the chromatographic separation is finished and the fluorescence detector records the oxidation products as a sum [59].

Various PSP toxins exhibit different fluorescence intensities after their oxidation, which must be taken into account in the evaluation of the chromatograms for the determination of PSP toxicity (Table 3).

2.1.2.2. Toxin separation

Many chromatographic techniques have been developed for separating PSP toxins in their underivatized form. At first these separations were carried out using ion-exchange and/or gel permeation techniques [60–62] and later silica-based HPLC-columns (amino or cyano columns) were applied [21,39,59]. However, the breakthrough in HPLC for PSP separation was the introduction of ion-pair chromatography [63].

Sullivan and Wekell [64] utilized an HPLC system equipped with a postcolumn derivatization unit and a fluorescence detector. HPLC separation of the PSP toxins is carried out on a polystyrene–divinylbenzene resin column (PRP-1; Hamilton, Darmstadt, Germany) and gradient elution with phosphate buffer solution, containing heptane and hexanesulphonic acids as ion-pair reagents. The carbamate toxins are well separated. However, the separation of the N-sulphocarbamoyl toxins C1–C4 is poor (Fig. 6).

TABLE 3

RELATIVE FLUORESCENCE INTENSITY OF PSP TOXINS AFTER OXIDATION UNDER ALKALINE CONDITIONS [53]

Toxin	Relative fluorescence intensity	Relative toxicity
Saxitoxin	1.00	1.00
Neosaxitoxin	0.04	1.00
Gonyautoxin I	0.05	0.73
Gonyautoxin II	1.80	0.42
Gonyautoxin III	1.80	0.67
Gonyautoxin IV	0.05	0.27
B1	0.41	<0.05
B2	0.05	0.09
C1	0.48	0.06
C2	0.48	0.02

When decarbamoyl toxins are present in the samples the chromatograms are ambiguous. Especially the separation of dc-STX and STX is not possible by application of HPLC according to Sullivan and Wekell [64] (Fig. 7). Therefore, it became an urgent issue to improve HPLC methods that would permit the complete separation of carbamate and decarbamoyl toxins [57].

Oshima *et al.* [66] proposed the application of three chromatographic runs for PSP determination. Three groups of toxins categorized by their basicity (group I, C1–C4; group II, GTX I–IV, B1 and B2, dc-GTX I–IV; group III, NEO, dc-STX, STX) are separated in three HPLC systems under isocratic conditions. A reversed-phase column (Develosil C_8-5; Nomura Chemicals) is used, and the eluents for carbamate–decarbamoyl separation contain heptanesulphonic acid as ion-pair reagent and tetrabutylammonium phosphate for the separation of the N-sulphocarbamoyl-11-hydroxysulphate toxins (because of their acidic nature). The method avoids gradient elution, and reliable results are obtained [67]. However, the expensive HPLC equipment and time-consuming prechromatographic steps are serious drawbacks of this method. Therefore, at pres-

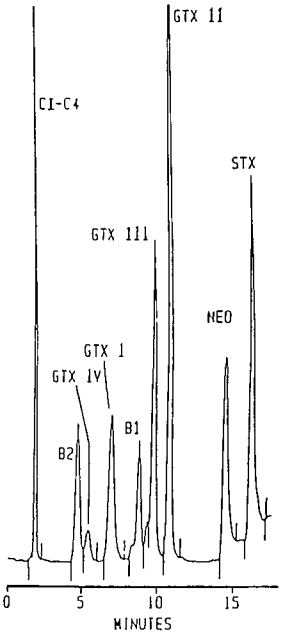


Fig. 6. HPLC illustrating the separation of PSP toxins by the method of Sullivan and Wekell [64].

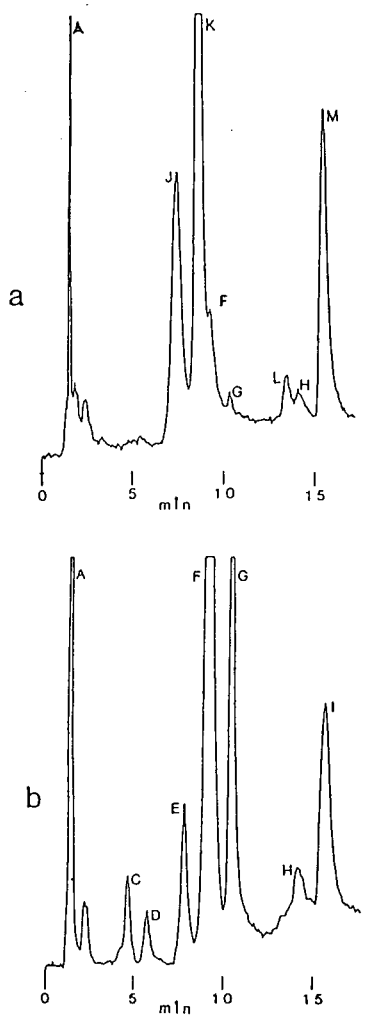


Fig. 7. HPLC of PSP toxins in shellfish extracts according to the method of Sullivan *et al.* [20]. (a) Littleneck clams (*Protothaca staminea*); (b) mussels (*Mytilus edulis*). A = C1 and C2; C = GTX IV; D = GTX I; E = B1; J = dc-GTX III; K = dc-GTX II; F = GTX III; G = GTX II; L = dc-NEO; M = dc-STX; I = STX.

ent, HPLC with gradient elution according to Sullivan and Wekell [64] is the most widely applied method for PSP determination [37,68].

Luckas *et al.* [57] proposed ion-pair chromatography on an RP-C_{18} column (Nucleosil 7-C_{18}; Macherey–Nagel, Düren, Germany) with octanesulphonic acid in the phosphate buffer eluent and isocratic elution to overcome the problem of dc-STX–STX separation. As the application of this method led to interferences in the chromatograms at the retention times of gonyautoxins, the chromatographic conditions were modified: the application of an RP-C_{18} column and two phosphate buffers containing octanesulphonic acid and acetonitrile–tetrahydrofuran as eluent was proposed [69]. A two-step elution allows the separation of carbamate and decarbamoyl toxins, and good resolution of the more strongly retained toxins (NEO, dc-STX, STX) is achieved (Fig. 8).

The separation dc-STX and STX is important because in shellfish N-sulphocarbamoyl toxins are metabolized into carbamate toxins in a first step [70], then the carbamate toxins are partially converted into decarbamoyl toxins, especially into dc-

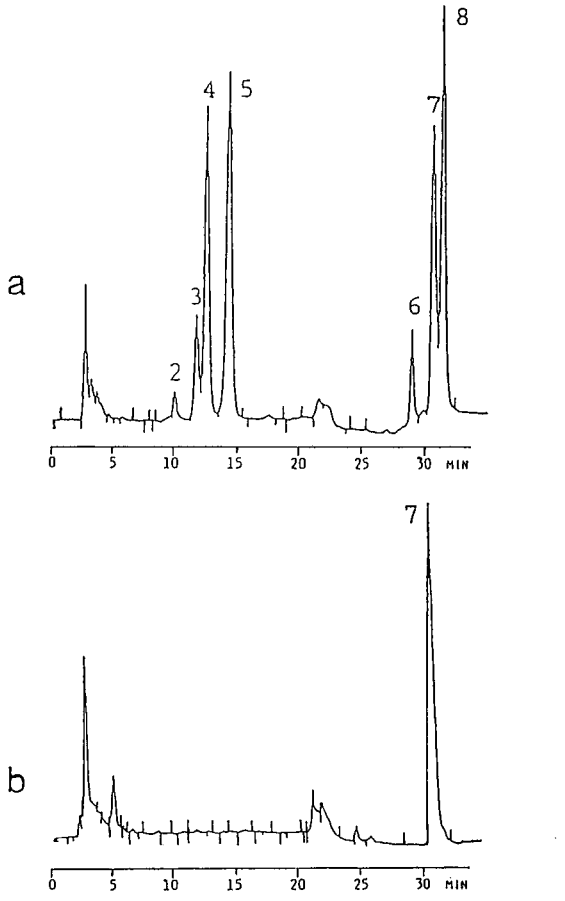


Fig. 8. HPLC of PSP toxins in shellfish extracts according to the method of Thielert *et al.* [69]. (a) Fresh mussels (*Mytilus edulis*, Spain); (b) canned mussels (*Mytilus edulis*, Spain). 2 = dc-GTX II; 3 = dc-GTX III; 4 = GTX II; 5 = GTX III; 6 = NEO; 7 = dc-STX; 8 = STX.

STX [21,71]. Therefore, in seafood carbamate toxins in additon to decarbamoyl toxins, especially dc-STX, have to be determined [16].

Thielert *et al.* [69] proposed extracting the samples with 0.03 *M* acetic acid to avoid destruction of the toxins. After ultrafiltration the extracts are injected directly into the chromatograph. To support the data, aliquots of the solution are treated with hydrochloric acid. The N-sulphocarbamoyl toxins are thus converted into their corresponding carbamate toxins, and higher concentrations of carbamate toxins, measured after HCl treatment, are good indicators of the N-sulphocarbamoyl content of the samples. Additionally, the calculation of the sum of STX compounds (*i.e.*, STX, GTX II and GTX III) and OH-STX compounds (*i.e.*, NEO, GTX I and GTX IV) makes it possible to typify PSP producers [72–75].

Irrespective of the PSP determination method applied, the sample preparation plays an important role in obtaining reasonable results. Obviously, differing data obtained with the mouse bioassay and HPLC may be explained by partial destruction of some toxins by the extraction procedure with 0.1 *M* HCl, which is recommended for both the mouse bioassay [43] and HPLC [64]. In addition, the inaccurate determination of decarbamoyl toxins by HPLC [16,39,40,53] may lead to disagreements between the methods. The influence of pH, temperature and storage time of the extracts on the variability of the mouse bioassay is well known [49], whereas the effect of acid treatment and heating time during the extraction process has been studied only recently [76]. Based on these data, it has been proposed to mix a homogenate with an equal volume of 1.0 *M* HCl, to heat in boiling water for 5 min and to apply the supernatant for PSP determination.

Among the advantages of the HPLC methods over the bioassay are greater sample throughput and significantly better sensitivity, in addition to the ability to determine individual PSP toxins (Table 4). Therefore, HPLC methods appear to be a viable alternative to bioassays for PSP determination in seafood.

TABLE 4

DETECTION LIMITS FOR PSP TOXINS BY HPLC AND MOUSE BIOASSAY [20]

Toxin	Detection limit (μM)	
	HPLC[a]	Mouse bioassay[b]
B1	0.040	6.7
B2	0.150	6.7
C1	0.006	59.0
C2	0.006	3.9
GTX I	0.100	0.5
GTX II	0.006	1.0
GTX III	0.006	0.6
GTX IV	0.100	0.5
NEO	0.065	0.4
STX	0.014	0.5

[a] Based on a 20-μl injection; twice baseline noise.
[b] Based on absolute toxicities for each toxin.

2.1.3. Mass spectrometry and HPLC–MS

Mass spectrometry (MS) is a powerful technique that also has an important future for the analysis of marine toxins. In additon to high sensitivity and selectivity, MS can provide structural information useful for the confirmation of toxin identity and the identification of new toxins [77]. Fast atom bombardment (FAB) MS has been investigated for PSP toxins and has proved useful as a means of structural confirmation at moderate sensitivity [78–80]. Recently, Quilliam *et al.* [81] have shown that HPLC–ion-spray MS is an excellent technique for the analysis of marine toxins. The ion formation mechanism is based on the ion evaporation phenomenon [82]. The technique can be used with a wide range of flow-rates and thus can be used on-line with HPLC and for direct sample introduction. PSP toxins are also well suited to ion-spray MS, whereby an abundant $[M+H]^+$ ion is observed.

However, combined HPLC–MS of PSP toxins has proved more challenging. Unfortunately, alkanesulphonates used as ion-pair reagents in the HPLC separation methods interfered with the ion-spray method. Therefore, various stationary phases and eluents are currently being investigated to improve the chromatographic separation [83]. HPLC separation on a PRP-1 column with an ammonium formate buffer and mass chromatograms of PSP toxins are illustrated in Fig. 9.

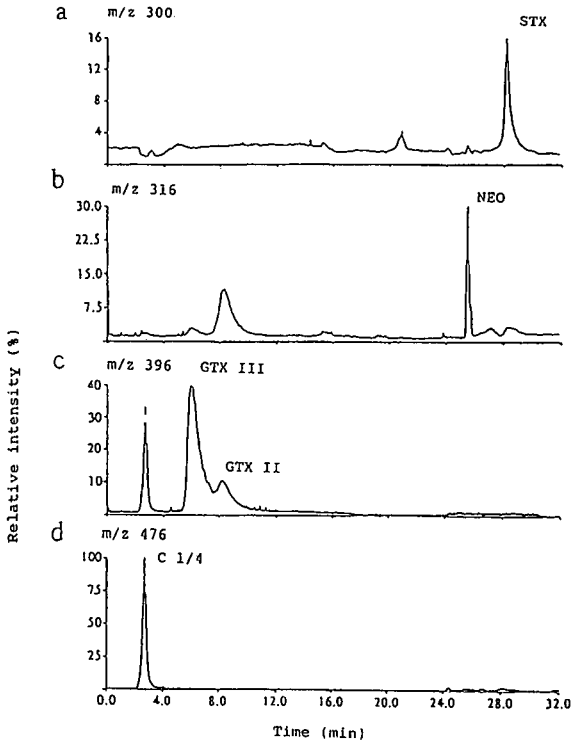


Fig. 9. Mass chromatograms from the HPLC–ion spray MS analysis of a mixture of PSP toxins [83].

2.2. *Methods for DSP determination*

Control measures to protect humans against DSP contaminated seafood are carried out by application of biological and chemical procedures [84]. The biological methods include the microscopy of certain algae, bioassays with mice and rats and immunological assays [*e.g.*, enzyme-linked immunosorbent assay, (ELISA)] [85]. Two bioassays have been extensively employed for control purposes, the mouse bioassay and the rat bioassay (Table 5). The mouse bioassay includes the i.p. injection of a purified extract. All DSP components are measurable. In the rat bioassay shellfish tissue is mixed with a normal diet and offered to the animals. The diarrhetic effects of OA, DTX-1, DTX-3 and in principle all compounds with similar effects are detected by this procedure. The chemical methods involve HPLC separation followed by fluorescence measurement. HPLC methods allow the sensitive determination of the individual DSP toxins OA and DTX-1. However, no collaborative study of any of the methods has yet been conducted, hence there are no internationally recommended procedures for DSP determination [28].

2.2.1. *Bioassays*

2.2.1.1. *Mouse bioassay*

The procedure consists in an extraction of shellfish soft tissue followed by i.p. injection into mice. The animals are observed for 24 h, the end-point being death. In cases of death, dilutions of the extract are tested in order to calculate the concentration of DSP, expressed as mouse units (MU) per gram or 100 g of shellfish. The procedure is unspe-

TABLE 5

METHODS FOR DSP DETERMINATION [84]

Country	Statutory limits	Methods of analysis
Denmark	No detectable amount	Mouse bioassay Rat bioassay
Germany	No detectable amount	Rat bioassay
France	0.2–0.4 MU/g digestive glands	Mouse bioassay
Ireland	No detectable amount	Rat bioassay, HPLC
Japan	5 MU per 100 g soft tissue	Mouse bioassay
Netherlands	No detectable amount	Rat bioassay
Norway	5–6 MU per 100 g soft tissue	Mouse bioassay
Portugal	No detectable amount	Mouse bioassay
Spain	No detectable amount	Mouse bioassay
Sweden	60 μg per 100 g soft tissue	HPLC, mouse bioassay

cific, as no signs except death are observed; the precision and sensitivity are unknown [86,87]. OA, DTX-1, DTX-3, PTX and YTX are detected, so a positive result does not necessarily mean the presence of a diarrhetic toxin.

2.2.1.2. Suckling mouse bioassay

Shellfish extracts are administered intra-gastrically to 4–5-day old mice using Teflon tubing, and the mice are kept for 4 h. After killing the mice, the whole intestine is removed and the fluid accumulation ratio (FAR) is expressed as the ratio of intestine mass to that of the remaining body. FAR values above 0.8–0.9 indicate a positive reaction. OA, DTX-1 and DTX-3 produce a positive reaction and PTX does not [88].

2.2.1.3. Rat bioassay

Soft tissue of shellfish (or digestive glands) is mixed with normal rat feed and offered to rats that have been starved for 24 h. After a 16-h period signs of diarrhoea and feed refusal signs are noted, and a semi-quantitative estimate of DSP toxicity is made based on these data. OA, DTX-1 and DTX-3 can be detected by this procedure. PTX and YTX give no reaction [26].

2.2.2. HPLC for DSP determination

In addition to the bioassays, many efforts have been made to determine DSP toxins OA and DTX-1 by HPLC. The HPLC method of Lee *et al.* [89] involves precolumn derivatization of the DSP toxins with 9-anthryldiazomethane (ADAM) and fluorescence detection. However, the application of the reaction with ADAM requires an additional clean-up step after derivatization. To avoid this time-consuming procedure the chromatographic equipment was modified by incorporating a column-switching system [90]. However, problems still arise from the instability of the ADAM reagent. Therefore, the reaction with 4-bromomethyl-7-methoxycoumarin (Br-Mmc) for derivatization of DSP toxins is proposed [91].

2.2.2.1. Sample preparation

The sample preparation procedure suggested by Lee *et al.* [89] can be improved. The first step consists in extraction of the homogenized sample with methanol–water (80:20) followed by cleaning the raw extract with *n*-hexane. By application of Br-Mmc determination the purification with *n*-hexane can be omitted, but additional clean-up by solid-phase extraction (SPE) with silica gel must be carried out. After sample preparation the DSP toxins are dissolved in dichloromethane. This solution is suitable for both derivatizations, the esterification with ADAM and the reaction with Br-Mmc [92].

2.2.2.2. Derivatization procedures

2.2.2.2.1. Derivatization with ADAM. ADAM reacts with carboxylic acids to give the 9-anthrylmethyl esters (Fig. 10). The reaction is carried out without catalyst at room temperature in 60 min [93]. The resulting ADAM derivatives of OA and DTX-1 are detectable with high sensitivity by HPLC with fluorimetric detection (excitation at 365 nm, emission at 412 nm).

2.2.2.2.2. Derivatization with Br-Mmc. Br-Mmc has been extensively used as a label for the derivatization of acidic compounds [94,95]. A convenient one-vial procedure using Br-Mmc for esterification of OA and DTX-1 to give fluorescent coumarin esters (excitation at 325 nm, emission at 390 nm) has been developed [91]. The derivatization of the DSP toxins with Br-Mmc is performed with a crown ether as catalyst in alkaline solution (Fig. 11).

In contrast to the ADAM esterification, the substances used for the Br-Mmc reaction are stable; no interfering peaks due to the derivatization reagent appear in the chromatograms [92].

2.2.2.3. HPLC separation

2.2.2.3.1. HPLC of ADAM derivatives. Usually the fluorescent ADAM derivatives of OA and DTX-1 are separated after clean-up on silica gel by HPLC on a C_{18} reversed-phase column using acetonitrile–methanol–water (8:1:1) as eluent. Both DSP toxins are clearly separable. The retention of OA was about 15 min and that of DTX-1 about 24 min [89]. In Fig. 12 an example of the application of this method is given [96].

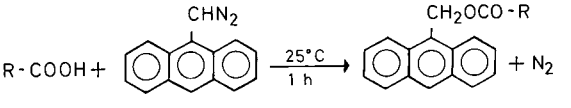


Fig. 10. Derivatization of OA and DTX-1 with ADAM [93].

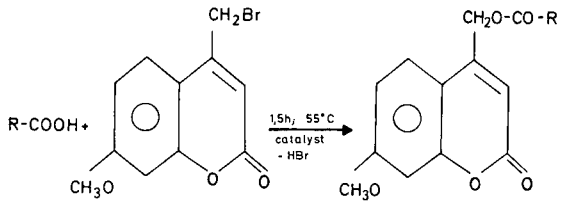


Fig. 11. Derivatization of OA and DTX-1 with Br-Mmc.

The purification of extracts containing ADAM derivatives is necessary to avoid interferences in the chromatograms. This procedure, however, performed by SPE, is time consuming and has negative effects on the reproducibility of data. To avoid the clean-up step, *i.e.*, to inject the derivatives directly into the chromatograph, the HPLC equipment was modified [90]. Two reversed-phase columns (column A, 25-cm RP-C_8; column B, 25-cm RP-C_{18}) and an enrichment column (5-cm RP-C_{18}) are combined with switching valves. After separation on the RP-C_{18} column A (pump A, eluent A) and cutting, either OA or DTX-1 is fixed on the enrichment column. By switching the valves again, the DSP toxins are eluted from the enrichment column and analysed by HPLC on the RP-C_{18} column B (pump B, eluent B) and subjected to fluorescence detection (Fig. 13). This method allows the injection of DSP-containing extracts into the chromatograph immediately after derivatization. In the chromatograms no interferences are visible and the toxin derivatives appear as sharp peaks (Fig. 14).

2.2.2.3.2. HPLC of Br-Mmc derivatives. For the HPLC of the Br-Mmc derivatives the use of a

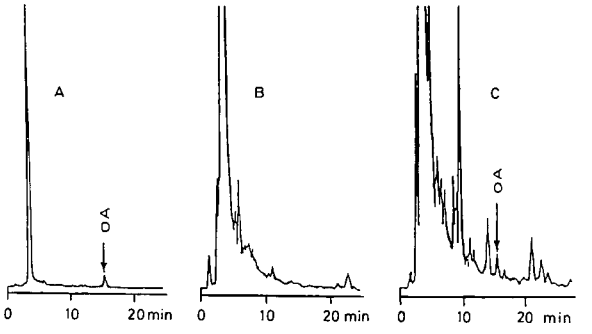


Fig. 12. HPLC of ADAM derivatives [96]: (A) okadaic acid; (B) uncontaminated mussels (Antifer, January 1988); (C) contaminated mussels (Antifer, August 1988).

C_{18} reversed-phase column and isocratic elution with acetonitrile–water (70:30) is suggested [91], and no interfering peaks appear at the retention time of the DSP toxins (Fig. 15). Br-Mmc derivatives of OA and DTX-1 are stable and an additional clean-up step after the derivatization is not required [92].

2.2.3. Mass spectrometry and HPLC–MS

Mass spectrometry using FAB ionization has been used by several workers for the mass spectral characterization of DSP toxins and the ADAM–OA derivative [97–99]. One report also included an attempt at liquid chromatography–mass spectrometry (LC–MS) coupled to a continuous-flow FAB interface [98]. Although a negative-ion chromatogram of the deprotonated molecular species of DTX-1 (*m/z* 817) was presented, the toxin eluted very early with a peak width of almost 5 min and no details of the sensitivity of the method were given [100]. In a more recent investigation, the presence of OA was confirmed by an impressive combination of electron impact (EI), chemical ionization (CI) and FAB mass spectrometry of the underivatized, and trimethylsilyl (TMS) and pentafluorobenzyl (PFB) derivatized toxin introduced via a direct probe [101].

Quilliam *et al.* [81] recently reported on an investigation of ion-spray mass spectrometry for the analysis of marine toxins, and concluded that this ionization process has great potential for the analysis of trace levels of polar marine toxins by mass spectrometry (and tandem mass spectrometry) combined with either direct flow injection or HPLC techniques.

Pleasance *et al.* [100] described the application of a combined LC–MS method using ion-spray ionization for the sensitive determination of OA in natural populations of dinoflagellates. Recently, Quilliam and Pleasance [83] reported a study of the confirmation of an incident of DSP in Eastern Canada. Analysis of whole mussel tissue extracts by combined LC–MS confirmed the presence of DTX-1 (Fig. 16).

Although HPLC–MS equipment is expensive, possibilities of automation can begin to justify such an investment if large numbers of samples can be analysed quickly with high-speed methods. For research studies, of course, the amount of informa-

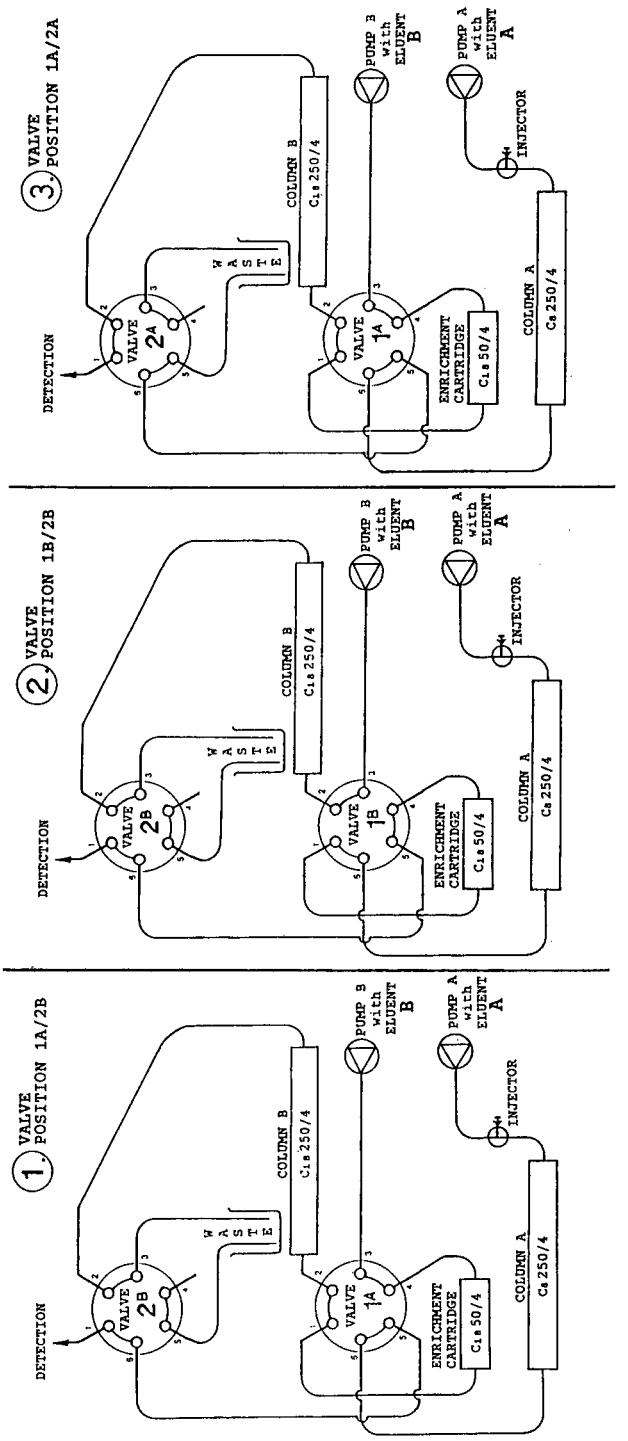


Fig. 13. Column-switching system for HPLC of ADAM derivatives [90].

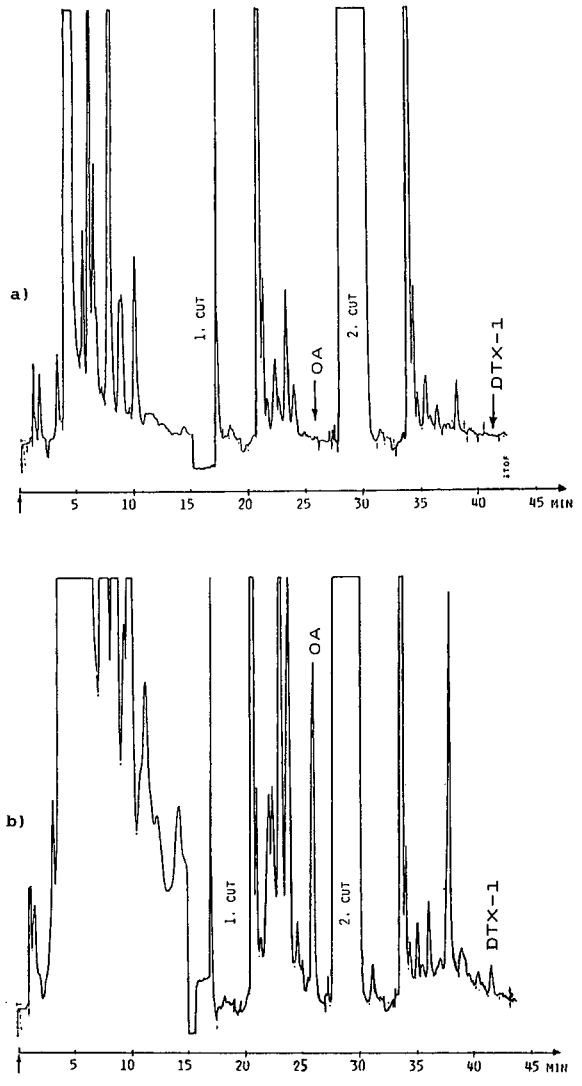


Fig. 14. HPLC of ADAM derivatives [91]. Column-switching system with two cuts. (a) Hepatopancreas (mussels, North Sea, uncontaminated); (b) hepatopancreas (mussels, Limfjord, contaminated).

tion provided by an HPLC–MS analysis is unsurpassed and will facilitate a greater understanding of the chemistry and biochemistry of seafood toxins.

3. CONCLUSIONS

Numerous cases of seafood poisoning occur worldwide each year especially due to the consumption of shellfish contaminated with high levels of toxins produced by marine dinoflagellates. As such incidents present a serious threat to public health and to the economy, there is a need for a better understanding the chemistry and biochemistry of seafood toxins and to develop analytical methods in order to guarantee safe, high-quality seafood products.

The most effective preventive measure is a monitoring programme with control at the source of the harvesting area. Production areas should be closed to harvesting when the toxin level in the shellfish approaches the established guideline or tolerance.

The best known hazard is paralytic shellfish poisoning (PSP). The accepted public health guideline recognized by most countries is 80 μg of PSP per 100 g of shellfish meat, using the AOAC mouse bioassay. This mouse bioassay has an acceptable

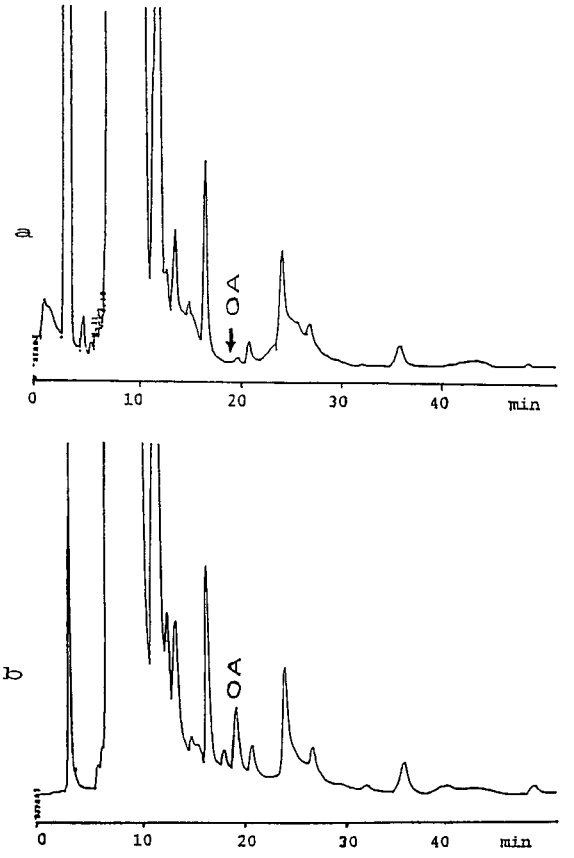


Fig. 15. HPLC of Br-Mmc derivatives [91]. (a) Hepatopancreas (mussels, North Sea, uncontaminated); (b) hepatopancreas (mussels, Limfjord, contaminated).

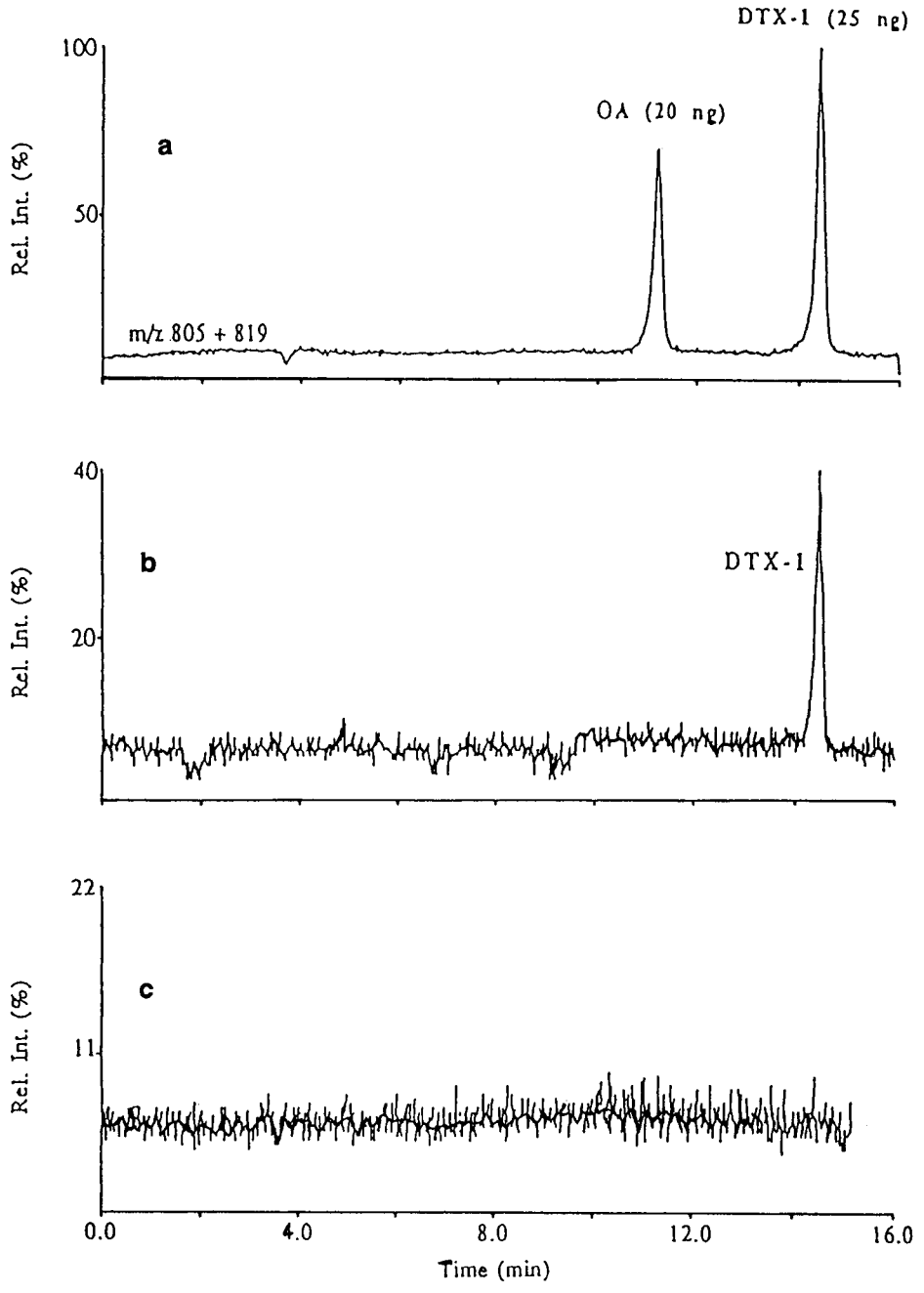


Fig. 16. LC–MS analysis of (a) DSP toxin standards and the extract of (b) suspect and (c) control mussel tissue extracts [83].

precision, but is lacking in sensitivity; 40 μg of PSP per 100 g shellfish meat are detectable. However, HPLC methods have been developed which can detect individual PSP toxins below the 1 μg per 100 g level.

Diarrhetic shellfish poisoning (DSP) is a more recently recognized problem. Okadaic acid (OA) and its derivatives (DTX-1 and DTX-3) are the principal toxins responsible for the diarrhetic symptoms.

The bioassays for DSP determination are not quantitative, the detection limit being 10 μg per rat. Compared with the bioassays, the HPLC method with fluorimetric detection for DSP provide advantages in terms of rapidity, accuracy, specificity and sensitivity.

The application of HPLC to the determination of the acidic components of DSP complex allows the detection of 10 μg of OA and/or DTX-1 per 100 g of shellfish meat. Therefore, HPLC analysis of these DSP components can act as indicators of DSP contamination, as no case of DSP contamination is known without the presence of at least one of these acidic components.

The acceptable levels for PSP and DSP differ significantly between countries. It is desirable for international organizations to evaluate the hazards caused by marine phycotoxins in order to provide a common basis for risk assessment, *i.e.*, to establish international toxin tolerances. For such an evaluation toxicity data are needed based on reliable analytical methodology. The further development of analytical methods for marine phycotoxins is especially needed as the enforcement of phycotoxin legislation is ultimately based on the ability of analysts to identify and determine these toxins accurately in seafood products. The HPLC methods discussed in this review are appropriate for solving these problems.

4. ABBREVIATIONS

Phycotoxins

PSP	Paralytic shellfish poisoning
B1–2, C1–4	N-Sulphocarbamoyl toxins
GTX I–IV	Gonyautoxin I–IV
dc-GTX I–IV	Decarbamoylgonyautoxin I–IV
NEO	Neosaxitoxin
dc-NEO	Decarbamoylneosaxitoxin
STX	Saxitoxin
dc-STX	Decarbamoylsaxitoxin
DSP	Diarrhetic shellfish poisoning
DTX	Dinophysistoxin
OA	Okadaic acid
PTX	Pectenotoxin
YTX	Yessotoxin

Derivatization reagents

ADAM	9-Anthryldiazomethane
Br-Mmc	4-Bromomethyl-7-methoxycoumarin

Units

MU	Mouse units

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Review

Application of ion chromatography to the determination of inorganic anions in foodstuffs☆

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ABSTRACT

A review on the applications of ion chromatography (IC) to the determination of inorganic anions in foodstuffs is presented. The anions most commonly determined in food, *i.e.*, SO^{2-}_3, NO^-_3 and NO^-_2, and to a lesser extent Cl^-, Br^-, I^-, SO^{2-}_4, IO^-_3, BrO^-_3 and phosphate, are considered. In comparison with standard methods for the determination of anions in food products, chromatographic methods are rapid, sensitive and precise. They also have the advantage of determining several ions simultaneously. The separation may be achieved by conventional IC, by ion interaction chromatography or by ion exclusion chromatography. IC has also been applied to the determination of Br, I, N and S in foods after oxidation or combustion of samples and conversion into anionic forms.

CONTENTS

1. INTRODUCTION

Originally, ion chromatography (IC) was developed as a chromatographic method for inorganic ions, using an ion-exchange resin as stationary phase and conductivity detection [1]. Currently, the definition of IC has been broadened to include the determination of organic ions and other techniques, such as ion interaction chromatography (IIC) and ion exclusion chromatography (IEC), which are based on other separation mechanisms rather than ion exchange, combined with nearly all of the HPLC (electrochemical or optical) detection systems. The stationary phases normally used in the ion-exchange mode are chemically modified polymers or silica gels with ionic functional groups,

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while the reversed phases widely used in HPLC are frequently employed for IIC separations. In IEC the separating material is a high-capacity cation-exchange resin in the hydronium form [2–6].

Ion chromatography has rapidly become a standard technique in water analysis [7,8], and it has also been demonstrated to be a very suitable technique for the solution of environmental problems [9–11] and in food analysis [12–14]. This paper reviews the applications of IC to the determination of inorganic anions in food products. The anions most commonly determined in food are SO_3^{2-}, NO_3^- and NO_2^- and, to a lesser extent, Cl^-, Br^-, I^-, SO_4^{2-}, IO_3^-, BrO_3^- and phosphate.

2. SAMPLE PREPARATION

When developing analytical methods for complex food matrices, sample preparation may be of fundamental importance [6]. Aqueous samples often require very little sample treatment; this also applies more or less to many beverages, but not to solid food samples. The extraction of ionic species from solid samples prior to IC analysis can often be achieved by heating a mixture of the homogenized sample with water, extractant solution or eluent in a blender or an ultrasonic bath for a specified time. After the sample has been dissolved, a further clean-up stage is often essential before an injection can be made into the ion chromatograph. Clean-up methods may be as simple as a filtration step or an adjustment of the pH, or they may be more elaborate such as selective removal of the analyte from the sample or elimination of interfering matrix components.

In this stage of the sample preparation many foods present problems owing to the existence of protein matter in the extract, which makes it difficult to obtain a clear solution. Such interference can be eliminated by boiling the extract to denature the proteins, by adding an organic solvent (such as acetone or methanol) or by employing Carrez I {$K_4[Fe(CN)_6]$ solution} and Carrez II [$Zn(CH_3COO)_2$ in acetic acid] reagents, which are very common in food analysis. The small sample volume required for IC analysis, in comparison with other analytical methods, makes this clarification step easier. Subsequently a separation of particulate matter, by filtration with or without a previous centrifugation step, is normally performed. Ultrafiltration devices in which the sample is forced under pressure through a membrane can also be applied.

Another common interference is due to soluble organic matter. There are several approaches to eliminate organic matter, such as treatment with activated carbon, solvent extraction or even selective removal of the analyte from the sample using ion-exchange resins. Usually, when anions are determined in foodstuffs by IC, the last stage of sample preparation before injection frequently implies removal of organic material by passing the solution through a disposable solid-phase extraction cartridge that contains a hydrophobic stationary phase (*e.g.*, C_{18} or polymeric) [15] in tandem with a disposable 0.45- or 0.20-μm filter in a single operation.

3. SULPHITES

Sulphites have long been used as preservatives in foods. There are three functions performed by sulphiting agents (sulphur dioxide, sulphites, hydrogensulphites and metabisulphites) in food products: antimicrobial agent, antioxidant and browning inhibitor. The last action could be related to the prevention of enzymatic browning in fresh fruits and vegetables or to the control of non-enzymatic browning in processed food. Sulphite is known as one of the most effective inhibitors of non-enzymatic browning, and although its chemical mechanism is not fully understood it possibly involves interactions of hydrogensulphite with carbonyl compounds present in food.

Recently, the addition of sulphites to food has become an important safety subject because of a number of documented adverse reactions in hypersensitive individuals, especially asthmatics. This has caused government agencies to review its use and to issue new regulations. Thus, the US Food and Drug Administration (FDA) requires that the presence of sulphite at or above 10 ppm of SO_2 be declared on the label of food products.

Sulphite added to food is present in free or bound forms. Bound sulphite consists of reversibly and irreversibly bound forms. The reversibly bound sulphites may be released either by an alkali treatment or more slowly by distillation with acid, whereas irreversibly bound sulphites, which form very stable addition compounds, are not detected by most ana-

lytical techniques. The sum of free and reversibly bound sulphite is referred to as total sulphite.

Over the years, the Monier-Williams (M-W) [91] method became the standard method for the determination of sulphite in food products with which other methods were compared. Although numerous modifications have been made since its first presentation, it consists of an acid distillation of samples, liberating SO_2. The latter is collected in hydrogen peroxide solution and oxidized to H_2SO_4, which is determined either by titration or gravimetrically. The modified M-W method is time consuming, it is not applicable to certain foods containing volatile acids and organic sulphur compounds and is subject to interference at low sulphite levels. Further, it is not very sensitive; in fact, the 10 ppm level was chosen by the FDA because it is considered to be the limit of detection of the method. In the FDA-optimized M-W method [92] as little as 1 ppm of SO_2 in foods can be detected (provided that the interference level is low), but even though the contribution from interferences is minimal, the method is still slow because it requires distillation for 1.75 h and it is not suitable for rapid screening of multiple samples. Further, because the allergic reactions might be to free but not to bound sulphite, a more reliable analytical technique is needed that is able to distinguish between free and bound sulphite (the M-W method cannot).

Recently, rapid and sensitive instrumental methods have been proposed for determining sulphite in food products. Among them, those using flow-injection analysis [16], differential-pulse polarography [17] and especially IC methods must be emphasized. The different procedures described in the literature for determining sulphite by IC have been classified in three groups, as shown in Table 1.

The procedures included under acid distillation in Table 1 combine the time-tested M-W procedure for isolating SO_2 from a complex food sample (in a reduced time) with a sensitive and specific detection system provided by IC. The approach implies a 10-min sample distillation with H_3PO_4, followed by purging with nitrogen and collecting the SO_2 produced in different absorbing solutions that are then analyzed by IC. In order to avoid interferences from other volatile and oxidizable sulphur compounds, the SO_2 released may be collected and stabilized as sulphite in a non-oxidizing alkaline medium of formaldehyde and NaOH [18–20], or in NaOH solution alone although there is a greater risk of oxidation to sulphate [21]. Other methods use formaldehyde in an almost neutral medium of potassium hydrogenphthalate, which forms the hydroxymethylsulphonate adduct (HMS) which is subsequently separated by IC [22]. Procedures that collect the distilled SO_2 in H_2O_2 solution, as in the M-W method, and determine the SO_4^{2-} produced by means of IC are also included within this group [23,24].

Generally, most acid distillation techniques provide a sensitive determination of sulphite in many foodstuffs, the limit of detection being 1 ppm of SO_2. The previous separation of sulphite from the matrix caused by the acidic treatment makes the further chromatographic determination much easier and, therefore, the chromatographic systems described in the literature are all equally efficient. One objection may be the 10-min distillation which is apparently insufficient in some instances. Further, although two of the procedures showed the possibility of distinguishing between free and bound sulphite, the procedures for determining free sulphite were different, one using an identical technique to that for total sulphite (treatment with H_3PO_4), but without heating [19], and the other measuring the free SO_3^{2-} in a mixture of the sample with an alkaline solution (NaOH–HCHO) [18]. The time for the complete analysis is about 25 min.

Other techniques are based on the liberation of bound sulphite from food by an extraction with alkali, which is more rapid than acid distillation. The headspace liquid chromatographic procedure [25,26] uses the headspace technique to liberate SO_2 from the alkaline extract and ion chromatography to determine low ppm levels of sulphite. The technique involves converting free and reversibly bound sulphite to SO_3^{2-} by treating the sample with an extracting solution at pH 11. An aliquot of the extract is acidified with H_3PO_4 to convert the liberated SO_3^{2-} to SO_2, which fills the headspace. Finally, a portion of the headspace is sampled and the SO_2 is converted back to SO_3^{2-} in the sampling syringe, which contains a basic trapping solution that is then analysed by IC. The procedure is very efficient in avoiding matrix interferences, its limit of detection is 1 ppm of SO_2 and the comparison with the M-W method was concordant for most samples,

TABLE I

ION CHROMATOGRAPHIC DETERMINATION OF TOTAL SULPHITE IN FOODSTUFFS

Flow-rates and retention times are not included because they do not play an important role as a result of the previous sulphite treatment.

Treatment	Absorbing solution	Column	Eluent	Detection	Ref.
Acid distillation	HCHO–NaOH	Dionex AS-3	2.9–3.0 m*M* $NaHCO_3$–	Conductivity and amperometry (Ag, +0.4 V)	19[a]
		Dionex AS-5	2.2–2.4 m*M* Na_2CO_3	Conductivity	20[a]
		Dionex AS-3		Conductivity and amperometry (pulsed, Ag, +0.4 V)	21
	NaOH	Dionex AS-3		Conductivity	22
	HCHO–KHP (pH 6.1)	Nucleosil 10 Anion	10 m*M* phthalate (pH 6.1)	Indirect refractometry	23
	H_2O_2	Chrompack Ionsphere A		Conductivity	24
	H_2O_2		Phthalate (pH 5.7)	Indirect spectrophotometry (280 nm)	25
Alkaline extraction	Na_2HPO_4–mannitol–$FeSO_4$ (pH 11)	Hamilton PRP-X100	30 m*M* methanesulphonic acid (pH 11) (5% ACN)	Amperometry (glassy C, +0.6 V)	26,27[a]
	Na_2HPO_4–mannitol (pH 9)	Wescan anion-exclusion Brownlee Polypore Hion exclusion	5.0–20 m*M* H_2SO_4	Amperometry (Pt, +0.4 V/+0.6 V)	28–32[a]
	Na_2HPO_4–mannitol (pH 9)	Dionex ion-exclusion AS-1	10 m*M* H_2SO_4	Amperometry (pulsed, Pt, +0.7 V)	33
	HCHO–NaOH	Dionex AS-3	2.9 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Conductivity and amperometry (pulsed, Ag, +0.4 V)	21
Non-alkaline extraction	H_2O	Dionex AS-3	2.9 m*M* $NaHCO_3$–2.2 m*M* Na_2CO_3	Conductivity and amperometry (pulsed, Ag, +0.4 V)	21
	10% ethanol	Hamilton PRP-X100	30 m*M* methanesulphonic acid (pH 10.8) (5% ACN)	Amperometry (glassy C, +0.6 V)	34
	HCHO (pH 5.1)	Brownlee Polypore Hion exclusion	10 m*M* H_2SO_4	Amperometry (Pt, +0.7 V)	35
	HCHO (pH 5)	Spherisorb 10 ODS Altex Ultrasil Octyl	52 m*M* TBA–50 m*M* acetate (pH 5.8)	Spectrophotometry after post-column reaction (412 nm)	36
	HCHO (pH 5)	Zorbax ODS	5 m*M* TBA–50 m*M* acetate (pH 4.7)	Spectrophotometry after post-column reaction (412 nm)	37

[a] Free sulphite may also be determined.

except for some vegetables with a natural sulphite content and other foods for which the alkaline treatment liberates additional sulphite that the acid distillation does not. Nevertheless, the method is laborious and not as simple as other IC techniques which are more direct.

The fastest determination of SO_3^{2-} in the alkaline extract involves ion exclusion chromatography (IEC) with amperometric detection [27–33]. Separation into an anion exclusion column (frequently sulphonated styrene–divinylbenzene) is achieved by a combination of mechanisms such as Donnan exclusion, partitioning and size exclusion. Among the various components of the extract, strong anions are repelled by the negatively charged groups of the resin and the cations are retained on the column. The determination of organic acids (weaks acids) is probably the most common use of IEC. Weak acids are eluted in order of increasing pK_a values, hence the property of H_2SO_3 as a weak acid is utilized in this chromatographic technique. The method is rapid; the whole analysis can be carried out within 10 min, with a limit of detection of 0.1 ppm of SO_2, and it is also selective (see Fig. 1). The only major food component that behaves like sulphite in this system is ascorbic acid, although a baseline separation may be achieved by choosing a suitable mobile phase (6 or 20 m*M* H_2SO_4 is used as eluent). As for the headspace technique, this alkali extraction does not detect naturally occurring sulphite in some foods, and also does not effectively release sulphite bound to certain pigments produced in non-enzymatic browning reactions. The method can determine free sulphite (in this instance the extraction is accomplished in an acidic medium of pH 2) and total sulphite.

A direct alkaline–formaldehyde extraction procedure [20] has also been used in determining total sulphite, but the extract was very viscous and some difficulties also seem to exist in obtaining an adequate separation.

The non-alkaline extraction procedures include the extraction of sulphite in a neutral medium [20,34] or the stabilization of sulphite as the HMS adduct by reaction between SO_3^{2-} and formaldehyde in a slightly acidic medium [35–37]. One of these last techniques [35] is similar to the alkaline extraction–IEC determination described above [27–32], but HMS is very stable at pH 5.1 and, although the pH of the extract is rapidly adjusted by the eluent (10 m*M* H_2SO_4) in the chromatograph, it does not seem likely that under these conditions the sulphite may be completely liberated from the adduct. Water extraction [20] involves homogenization of samples with water, centrifugation and filtration of the supernatant. The aqueous extract is combined with an equal volume of CH_2Cl_2 and centrifuged again. An aliquot of the aqueous phase is injected into the column after a further clean-up stage. In the ethanol extraction procedure the sample is homogenized and extracted with 10% aqueous ethanol solution and injected after a C_{18} clean-up procedure. Both procedures use basic mobile phases that liberate the bound sulphite during the chromatographic run. It is worth emphasizing in this group the technique [36,37] based on the separation of the HMS adduct by IIC and further detection by means of a post-column reaction in two stages: first, SO_3^{2-} is liberated by treatment of HMS with alkali and then it reacts with Ellman's reagent and is detected spectrophotometrically. IIC uses a non-polar column in conjunction with an ion interaction reagent (the ionic modifier) in the mobile phase (in this instance tetrabutylammonium ion). The modifier acts as a movable site for ion exchange

Fig. 1. Chromatogram of sulphite determined by alkali extraction–IEC method. Dehydrated apple sample. The calculated concentration in the extract is 0.56 ppm of SO_2. Conditions: Wescan anion exclusion/HS column, eluent 20 m*M* H_2SO_4; amperometric detection (Pt, +0.6 V *vs.* Ag/AgCl). From ref. 31.

TABLE 2

SIMULTANEOUS CHROMATOGRAPHIC DETERMINATION OF NITRITE AND NITRATE IN FOOD SAMPLES

Column	Eluent	Detection	t_R (min) (NO_2^-/NO_3^-)	Sample	Ref.
Hamilton PRP-1	1.0 m*M* TPA fluoride (33% ACN) (1 ml/min)	Spectrophotometry (Conductivity)	10.5/13.3	Infant food, bacon and beer	38
Vydac 302 IC	11 m*M* CMS (pH 5) (2 ml/min)	Spectrophotometry (214 nm)	7.1/9.9	Cured meats	39
Waters CN Rad-Pak	1% Cetrimide–0.1 *M* KH_2PO_4 35% CH_3OH (1 ml/min)	Spectrophotometry (214 nm)	6.7/10.4	Cured meats	39
Waters NH_2 Rad-Pak	16 m*M* KH_2PO_4 (pH 3) (1 ml/min)	Spectrophotometry (214 nm)	6.5/8.4	Cured meats	39
Hamilton PRP-1	5.0 m*M* TPA bromide (33% ACN) (1 ml/min)	Spectrophotometry (240 nm)	7.4/11.1	Cured meats	40
Nucleosil 10 Anion	25 m*M* salicylate (pH 4.0) (1.4 ml/min)	Refractometry			41
Nucleosil 100 C_{18}	10 m*M* octylamine–H_3PO_4 (pH 4.0) (0.6 ml/min)	Spectrophotometry (210 nm)			41
Waters C_{18} Rad-Pak	Waters UV PIC A reagent (3 ml/min)	Spectrophotometry (214 nm)	2.5/4.0	Cured meats and vegetables	42
Ionosphere A	20–60 m*M* $NaClO_4$ (2 ml/min)	Spectrophotometry (209 nm)	3.6/4.2	Cooked ham	43
YEW SAX-1	15 m*M* $Na_2B_4O_7$–1 m*M* Na_2CO_3 (2 ml/min)	Spectrophotometry (210 nm) (Conductivity)	5.1/12.6	Vegetables	44
Waters IC-Pak A	5 m*M* K_2HPO_4 (pH 9.0) (0.8 ml/min)	Spectrophotometry (215 nm)	5.6/9.2	Cured meats	45
Biotronik Anion	5 m*M* CMS (pH 6.2–6.4) (1 ml/min)	Spectrophotometry (210 nm)	4.4/11.2	Cured meats	46
Vydac 300IC.405	Phosphate (pH 6.0)	Spectrophotometry (214 nm)		Cured meats	47

and it can be retained either on the resin alone (usually is a lipophilic ion) or as an ion pair with an ion of the sample (ion-pair chromatography).

Some of the non-alkaline extraction procedures are the simplest and may be of interest for screening purposes, but nevertheless none of them allows the determination of both free and total sulphite.

As has already been mentioned, some of the procedures described for the determination of total sulphite may also be used to determine free sulphite by the same chromatographic methods but with some modifications in the process of extraction. Nevertheless, concerning the most suitable pH for extracting free sulphite without liberating bound sulphite at the same time, there are some contradictions among the procedures described.

4. NITRATES AND/OR NITRITES

Nitrate salts are naturally present in many foods, mainly in vegetables, in concentrations that are characteristic of every species, although it also depends on the conditions of fertilization. The accumulation of large amounts of nitrate in plant tissues grown on heavily fertilized soils is of concern, particularly in infant food preparation, because the reduction of nitrate to nitrite in the infant intestine (more vulnerable because of its lower acidity), with subsequent absorption, could lead to cyanosis due to methaemoglobin formation. The potassium and sodium salts of nitrite and nitrate are also commonly used in the food industry, in curing mixtures for meats to develop and fix the colour, to inhibit the growth of microorganisms and to produce characteristic flavours. Recently, NO_2^- has been shown to be involved in the formation of low, but possibly toxic, levels of nitrosamines, many of which are potent carcinogens. Furthermore, NO_3^-, although not very toxic, can, under reducing conditions, be converted into NO_2^-. Therefore, the determination of nitrate and nitrite in foodstuffs has become increasingly important because of concern over their excessive dietary intake.

Traditionally, both ions have been determined in foods by spectrophotometric methods, the most common one involving diazotation of NO_2^- with sulphanilamide followed by coupling with N-(1-naphthyl)ethylenediamine. NO_3^- is previously reduced to NO_2^-, usually with Cd, and is then determined by difference. The spectrophotometric methods are time consuming, not very selective and can be unreliable for some samples. In addition, trace levels of nitrite in foods are often not detectable. In comparison, chromatographic methods are rapid, sensitive and precise. They also have the advantage of determining both NO_3^- and NO_2^- simultaneously [38–47]. This is of special concern in the analysis of meat products (see Table 2).

The literature on the application of IC to the determination of these anions shows nearly all the possibilities of this technique. The different modes may be related to the type of column used, *i.e.*, to the nature of the stationary phase. With respect to conventional IC, applications have been described that use silica- [39,41,43,48–52], poly(styrene–divinylbenzene)- (PSDVB) [46,53–55] or polymethacrylate-based (PM) [45,56] ion exchangers. Nitrates and nitrites can also be determined by IIC, employing octadecylsilyl (C_{18}) [41,42,57], octylsilyl (C_8) [58], PSDVB [38,40] or even cyano (CN) columns [39]. Nitrates, but not nitrites, which are eluted in the back of the solvent front, may be determined in a similar way using an amino-bonded column [59,60].

Ion interaction methods using C_{18}, CN or PSDVB columns give satisfactory results with standard solutions, but when the methods were applied to difficult samples, such as many meat products, poor separations were obtained. Irreproducible retention times and problems due to differences in matrix interferences as a result of variations in raw materials and/or processing methods were often found [39,40,42,45]. In contrast, excellent resolution of NO_2^- and NO_3^- is obtained with IC methods employing silica- or polymer-based columns, and the methods have been shown to be free from interferences (see Figs. 2 and 3). Linear calibration graphs have been obtained for nitrite and nitrate over a wide range, the detection limits being 2–3 ng [39,45].

Concerning detection systems, in most applications UV spectrophotometric detection is preferred [38–49,52,57–59], although other methods have also been reported, *e.g.*, conductivity [38,51,53–56], amperometry (NO_2^- only) [50,61] and refractometry [41]. UV detection is especially indicated in the selective determination of NO_2^- and NO_3^- in cured meats (which contain a large excess of chloride).

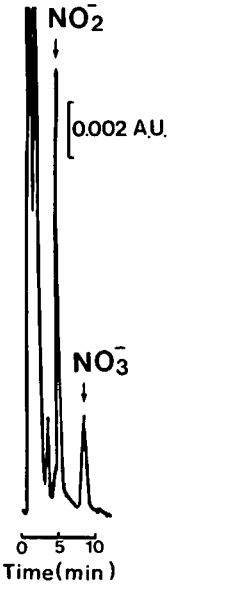

Fig. 2. Separation of nitrite and nitrate. Corned beef sample. Conditions: Waters IC-Pak A column with 5 m*M* dipotassium phosphate at 0.8 ml/min; UV detection (215 nm). The calculated concentration in the aqueous extract corresponds to 57 mg of nitrite and 32 mg of nitrate per kg corned beef. From ref. 45.

Recently, an IEC method with amperometric detection was proposed for the determination of NO_2^- alone [61]. In IEC most anions are eluted in the void volume and do not interfere with NO_2^-. As nitrite has a high pK_a value and is eluted late from the column (it depends on the column employed, but t_R was about 8 min on a Wescan anion-exclusion/HS column), the possibility of interference from other compounds as ascorbic acid or sulphite, which are eluted earlier, is minimal (see Fig. 4). Further, amperometric detection offers additional selectivity by selection of the operating potential. The limit of detection was 0.1 ng, which is one order of magnitude lower than that achieved by IC with UV detection.

Other applications concern the simultaneous determination of NO_3^- and other anions such as Cl^-, Br^-, SO_4^{2-} and PO_4^{3-} (occasionally NO_2^- is also determined), most of them by conventional IC, in different foodstuffs, mainly vegetables [51,52,54–56,60]. In vegetable analysis, interference from organic acids has frequently been observed. Peaks arising from malic, tartaric or glycolic acid may overlap the NO_3^- peak. In some instances this can be overcome by modifying the eluent [56], but in others it is necessary to use more specific procedures. These applications are shown in Table 3.

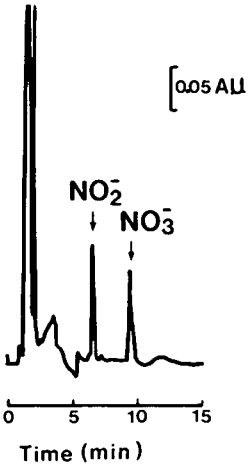

Fig 3. Separation of nitrite and nitrate. Bacon sample. Conditions: Vydac 302 IC column with 11.0 m*M* chloromethanesulphonic acid (pH 5.0) at 2 ml/min; UV detection at 210 nm. From ref. 39.

5. OTHER ANIONS

The determination of iodine in foods is important because, although it is an essential micronutrient, high levels of iodine in the diet may lead to thyroid-related problems. Because of the low levels at which it may be present and because losses of the element occur during sample digestion, a reliable determination of iodine in foods is very difficult. Milk and dairy products represent the main contribution, in

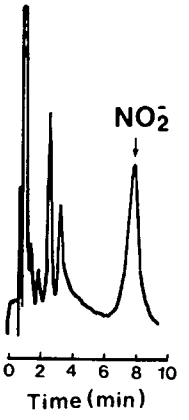

Fig. 4. Ion exclusion chromatographic determination of nitrite in ham. The calculated concentration in the extract is 0.25 ppm of nitrite. Conditions: Wescan anion exclusion/HS column, 20 m*M* H_2SO_4 at 0.8 ml/min; amperometric detection (Pt, + 1.0 V *vs.* Ag/AgCl reference). From ref. 61.

TABLE 3

ION CHROMATOGRAPHIC DETERMINATION OF NITRATE AND OTHER IONS IN FOODSTUFFS

Column	Eluent	Detection	t_R (min)	Other ions: t_R (min)	Sample	Ref.
Waters μBondapak NH_2	10 g/l KH_2PO_4 (pH 3.0) (1 ml/min)	Spectrophotometry (210 nm)	7.9	Br^- (6.4)	Vegetables, rice, cheese and flour	60
Dionex AS-3	2.1 m*M* $NaHCO_3$–1.68 m*M* Na_2CO_3 (3 ml/min)	Conductivity	9.2	NO_2^-, Cl^- (3.2), PO_4^{3-} (6.3)	Meat extract	54
Dionex AS-1	3.0 m*M* CO_3^{2-}–2 m*M* OH^-	Conductivity		SO_4^{2-}, PO_4^{3-}	Meat extract	55
Zipax SAX	0.5 m*M* phthalate	Indirect spectrophotometry (240 nm)	5.9	SO_4^{2-} (13.6), Cl^- (2.4)	Vegetables	52
Wescan 269-001 anion	4.4 m*M* phthalate (pH 3.9) (2 ml/min)	Conductivity	11.0	NO_2^- (7.4), Cl^- (5.8) PO_4^{3-} (pH 3.9) SO_4^{2-} (29.9)	Vegetables and salads	51
BAKC-1 (laboratory packed)	1.5 m*M* gluconic acid–1.5 m*M* boric acid (1.0 ml/min)	Conductivity	2.8	Cl^- (1.4)	Vegetables	56

the iodide form, to the dietary intake of iodine. Another important source of iodine is iodated table salt.

Various instrumental methods have been applied to the determination of iodide in milk, using either differential-pulse polarography [62,63], gas chromatography after conversion into an organic derivative [64,65], or iodide-specific electrodes [66]. The determination of iodide in milk and/or iodated table salt by IC, always with amperometric detection, has also been recommended [14,67,68]. Three different IC systems have been compared for the determination of iodide in milk sample extracts. Although all three columns performed well with standard solutions, there are significant differences when real samples are analysed. The best results were obtained with a Vydac 302 IC column (Separations Group) [68], because it accomplished a better separation of the I^- peak from other sample peaks (see Fig. 5). The method is sensitive, the detection limit for the food studied being about 25 μg/l of I^-. Iodine occurring in table salt may be in the iodide form, but also in the iodate form, and so it can be determined by chromatographic methods. Iodate has been determined either directly by IC [14] or by difference after first reducing iodate to iodide, which is then determined by IIC [69] (see Table 4).

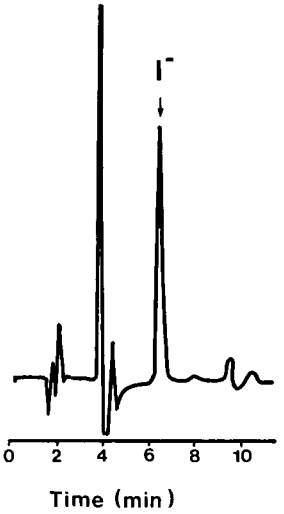

Fig. 5. Ion chromatographic determination of iodide in a whole milk extract. Conditions: Vydac 302 IC column, 6.5 m*M* KH_2PO_4 (pH 6.3) at 2 ml/min; amperometric detection (Ag, +0.155 V). From ref. 68.

Another application relates to the determination of bromate in bakery products [70–73]. BrO_3^- is employed as a flour bleaching agent and dough improver. In all the examples described the interference of Cl^- and procedures for avoiding it are mentioned. Chlorite, another bleaching agent used in the manufacture of candy products, has been determined by IC with UV detection [74] (see Table 4).

Ion chromatographic determinations also have been published for several ions in different beverages [23,33,37,57,75–82], especially brewery products [23,33,75–78]. Other interesting IC applications to the determination of Br^- in vegetables [83] and the determination of Cl^-, HPO_4^{2-} and SO_4^{2-} in various sugar foods have also been proposed [84].

6. ELEMENTS AND ANIONS

IC has been applied to the determination of Br, I, N and S in different foodstuffs after oxidation or combustion of samples and conversion of these elements, normally in organic form, into Br^-, I^-, NO_3^- and SO_4^{2-}, respectively.

Certain vegetable oils contain sulphur (and nitrogen) compounds, probably isothiocyanates and thiocyanates arising from the hydrolysis of other components present in seeds. Fish oils have also been shown to contain sulphur compounds, including sulphides and methylthio esters, considered to be derived from bacterial degradation of methionine. Although these sulphur compounds occur in trace amounts, in many instances they are responsible for characteristic odours and have attracted considerable attention in the recent past, because they are known to inhibit the catalytic hydrogenation reactions of oil hardening processes.

Chromatographic procedures for the determination of sulphur in oil and grease involve the combustion of samples in an oxygen bomb in such a way that all forms of sulphur are converted into SO_4^{2-}. After the combustion, the interior surfaces of the bomb were rinsed with the chromatographic eluent and, after a further clean-up stage, the purified solution was injected into a Waters IC Pak A column and detected by conductivity [85] or indirect UV spectrophotometric detection [86]. The method is rapid and sensitive, the detection limit being 0.1 ppm of S with spectrophotometric detection (phthalate eluent, pH 6.5) or 0.5 ppm with con-

TABLE 4

ION CHROMATOGRAPHIC DETERMINATION OF LESS COMMON IONS IN FOODSTUFFS

Ion	Column	Eluent	Detection	t_R (min)	Sample	Ref.
I^-	Dionex AS-2	40 m*M* $NaNO_3$–4 m*M* HNO_3 (2.6 ml/min)	Amperometry (Pt, +0.8 V)	2.0	Table salt	67
	Nucleosil 10 CN	CTMABr–phosphate (CH_3OH)	Amperometry (glassy C, +1.0 V)	13	Milk	14
	Vydac 302 IC	6.5 m*M* KH_2PO_4 (pH 6.3) (2 ml/min)	Amperometry (Ag, +0.155 V)	6.2	Dairy products and table salt	68
	Partisil 10 SAX	6.5 m*M* KH_2PO_4 (pH 6.2) (2 ml/min)	Amperometry (Ag, +0.155 V)	6.0	Dairy products and table salt	68
	Waters IC Pak Anion	5 m*M* *p*-hydroxybenzoic acid (pH 10.5) (0.9 ml/min)	Amperometry (Ag, + 0.155 V)	6.1	Dairy products and table salt	68
IO_3^-	Nucleosil 7 C_{18}	1.66 ml/l octylamine–0.4 g/l Na_2SO_4 (pH 6.0) (1.1 ml/min)	Spectrophotometry (230 nm)		Table salt	69
	LiChrosorb-NH_2	3.6 g/l NaCl–HCl (pH 3.9)	Spectrophotometry (205 nm)	6.42	Table salt	14
	LiChrosorb RP-18	4 g/l HDTMACl–1 g/l NaCl	Spectrophotometry (215 nm)	8.52	Table salt	14
BrO_3^-	Dionex AS-1	3.5 m*M* tetraborate (117 ml/h)	Conductivity	28	Bread	70,71
	Bio-Gel TSK IC anion PW	7 m*M* CMS (pH 5.5) (1.4 ml/min)	Spectrophotometry (205 nm) and conductivity	2.7	Bakery products	72
	Zipax SAX	0.25 m*M* phthalate (pH 4.3) (0.8 ml/min)	Indirect spectrophotometry (230 nm)	5.8	Bread	73
ClO_2^-	Oyobunko ASA400	Phthalate–borate (pH 6.7)	Spectrophotometry (250 nm)		Candy products	74

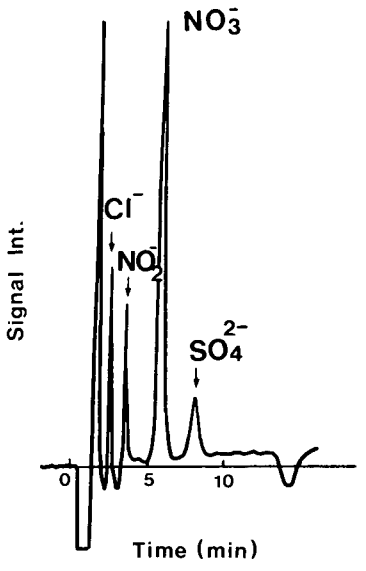

Fig. 6. Chromatogram of a fish oil sample after combustion. Conditions: IC-Pak A column, 1.0 m*M* potassium hydrogenphthalate at pH 6.5; UV detection at 290 nm. From ref. 85.

ductivity detection (borate–gluconate eluent, pH 8.5).

The same technique has also been proposed for the determination of nitrogen, which is oxidized mainly to NO_3^- (and some NO_2^-) and is detected by indirect UV spectrophotometry; hence one can simultaneously obtain the S and N contents of an oil sample by IC (see Fig. 6). In this instance it is necessary to degas the sample before analysis in order to avoid interference from dissolved nitrogen [87].

Similarly, combustion methods for milk and milk chocolate samples [88] (conversion of iodine into I^-) and for cocoa powder samples, after extraction with CH_2Cl_2 and ashing of the extract to convert Br into Br^- [89], have been published. Both ions were then determined by IIC. Total Br has also been determined in crops, after ashing, by IC with UV detection [90].

7. CONCLUSIONS

Among the anion chromatographic determinations described, those giving the best results may be emphasized. Thus, the IEC determination of SO_3^{2-} in food with amperometric detection seems especially suitable and it may be recommended for most foods and beverages at all sulphite levels. A rapid, sensitive and selective analysis can be accomplished, also allowing the separation between free and reversible forms of sulphite. This does not apply to darkly coloured foods.

Regarding NO_3^- and/or NO_2^- determinations, the simultaneous determination of both ions, which can be performed by means of several chromatographic systems, should be emphasized. Nevertheless, more reproducible and better results are obtained for the determination of NO_3^- and NO_2^- in difficult samples (*e.g.*, meat products) by means of conventional IC rather than ICC. The determination of nitrate in food products, especially vegetables, may be achieved with excellent results using different separation and detection systems. Although with many of these nitrate determination methods the interference of organic acids, which give peaks near the NO_3^- peak was mentioned, this interference can often be eliminated without difficulty. If the objective is the selective and sensitive determination of NO_2^- alone, then IEC with amperometric detection is superior and more convenient than conventional IC with either conductivity or UV spectrophotometric detection.

Finally, the very sensitive IC determination of iodide in foodstuffs, mainly dairy products, should be noted.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
CMS	Chloromethanesulphonic acid
CTMABr	Cetyltrimethylammonium bromide
FDA	Food and Drug Administration
HDTMACl	Hexadecyltrimethylammonium chloride
HMS	Hydroxymethylsulphonate
HPLC	High-performance liquid chromatography
IC	Ion chromatography
IEC	Ion exclusion chromatography
IIC	Ion interaction chromatography
K_a	Acid dissociation constant
KHP	Potassium hydrogenphthalate
M-W	Monier-Williams

PM	Polymethacrylate
PSDVB	Poly(styrene–divinylbenzene)
PIC	Paired-ion chromatography
TBA	Tetrabutylammonium
TPA	Tetrapentylammonium
t_R	Retention time
UV	Ultraviolet

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Determination of tranquilisers and carazolol residues in animal tissue using high-performance liquid chromatography with electrochemical detection

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ABSTRACT

A multi-residue method for the determination of tranquiliser residues in animal tissue is described. The procedure may be used to determine residues of the tranquilisers acepromazine, azaperone, chlorpromazine, haloperidol, propionylpromazine, xylazine, the metabolite of azaperone, azaperol, and the β-adrenoreceptor blocking agent carazolol. Existing methods of analysis for tranquilisers are based on ultraviolet and fluorescence detection and have been used for pig kidney analysis. Determination in this method was by high-performance liquid chromatography with electrochemical detection in the screen mode. The enhanced selectivity offered by the electrochemical detector allowed determination in liver extracts, which often give rise to more interferences on chromatographic traces when using conventional methods of detection. The method offers up to a ten-fold improvement in limits of determination over methods using ultraviolet and fluorescence detection. Recoveries and coefficients of variation have been determined in the range 2–25 μg/kg in pig kidney and liver. This electrochemical detection method has been used to measure residues in routine surveillance programmes.

INTRODUCTION

Tranquilisers are administered to food-producing animals for a variety of reasons, which may result in residues in meat and meat products. The main categories of use are for sedation prior to handling or examining the animal, or to sedate an animal prior to transportation. It is the use of tranquilisers before transporting animals to slaughter which is most likely to result in residues entering the food chain, since they are administered only a few hours prior to this event and insufficient clearance time may have been allowed. Tranquilisers are used in this instance to minimise death and injury during transport, and to reduce stress. Stress is especially noticeable in pigs which have been bred to give lean meat, because they are more susceptible to this condition which gives rise to tough meat.

The β-adrenoreceptor blocking agents can be used to give effects similar to the neuroleptics. Amongst this class, carazolol is known to be used. The neuroleptics, or true tranquilisers act by blocking the dopamine receptor sites in the brain, which overload in times of stress. β-Adenoreceptor blocking agents act by slowing down the heart rate.

Some of the tranquilisers concentrate in the kidney and others in the liver. Kidney is the target organ for azaperone [1], azaperol [1] and xylazine [2] and liver the target organ for the phenothiazines [3,4]. Carazolol is reported in some places to concentrate in the liver [5], and in others, the kidney [4,6].

A recent review on the analysis of veterinary drug residues in edible animal products has been made by Shepherd [4] and covers methods available for the determination of these compounds in animal tissue. Early methods for the determination of individual or small groups of residues using liquid chromatography (LC) and gas chromatography (GC) have been summarised by Van Ginkel *et al.* [7].

Multi-residue methods for the determination of tranquilisers have been described by Keukens and

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Aerts [8] and by Van Ginkel *et al.* [7]. Both of these methods were evaluated in our laboratory, and best results were found using modifications of the extraction procedure used by Keukens and Aerts [8] and the high-performance liquid chromatographic (HPLC) prcedure used by Van Ginkel. *et al.* [7]. Both of these methods use UV detection for all compounds and additional fluorescence detection for azaperol, azaperone and carazolol.

Chlorpromazine has been detected in human plasma at therapeutic levels using electrochemical detection (ED) [9] and it was decided to investigate this method of detection as a potential confirmatory method of analysis.

EXPERIMENTAL

Samples were prepared using a modification of the extraction and clean-up method described by Keukens and Aerts [8]. The HPLC method used was a modification of that described by Van Ginkel *et al.* [7].

Standards

The analytical standards used were obtained as follows: acepromazine was a donation from Border Research (Dundee, UK); azaperol was purchased from Janssen Biotech (Olen, Netherlands), azaperone from Janssen Biochimica (Beerse, Belgium), carazolol from Boehringer Mannheim (Mannheim, Germany) and chlorpromazine, haloperidol, propriopromazine and xylazine from Sigma (Poole, UK).

Materials

All solid chemicals, sulphuric acid and ammonia solution used in the procedure were AnalaR grade and obtained from BDH (Poole, UK). Water was obtained from an in-house Elga purification system, and other solvents were HPLC grade and obtained from Rathburn Chemicals (Walkerburn, UK). Bond-Elut C_{18} solid-phase extraction cartridges were obtained from Jones Chromatography (Mid-Glamorgan, UK).

A 10% sodium chloride solution and a 0.01 *M* aqueous sulphuric acid solution were prepared. Acidic acetonitrile was prepared by the addition of 1 ml of 0.05 *M* sulphuric acid to 100 ml acetonitrile.

Amber vials (4 ml) and vial inserts (300 μl) were prepared by rinsing successively with concentrated ammonia solution, water and acetone. They were dried using a stream of nitrogen.

The HPLC eluent was prepared by mixing 0.77 g of ammonium acetate, 500 ml of acetonitrile and 500 ml of water. The solution was filtered through a 0.22-μm membrane filter and degassed before use using low pressure and an ultrasonic bath.

Procedure

An excess of pig kidney was cut into small pieces and was homogenised in a 100-ml polypropylene centrifuge tube. About 5 g were accurately weighed out into a 50-ml polyethylene centrifuge tube. To the homogenate were added 10 ml of acetonitrile, and the tube was shaken. The sample was then mixed thoroughly for 30 s on a vortex-mixer and sonicated for 3 min in an ultrasonic bath. The mixing and sonicating processes were repeated, and the samples centrifuged at 10 000 *g* for 20 min.

After centrifugation, 7.5 ml of the supernatant were added to 40 ml of 10% sodium chloride solution in a 100-ml polypropylene tube. A Bond-Elut C_{18} solid-phase extraction (SPE) cartridge was placed on a Vac-Elut evacuation chamber and activated successively with 5 ml of methanol and 5 ml of water. The extract was immediately added to a reservoir connected to the SPE cartridge and allowed to pass through slowly at a flow-rate of about 1 ml/min by applying an appropriate pressure. Care was taken to prevent the cartridge from drying out at this stage. When all of the sample had passed through the cartridge, it was flushed with 0.85 ml of 0.01 *M* sulphuric acid and dried with air. The analytes were eluted with 3.5 ml of acidic acetonitrile into a prepared 4-ml vial.

The vial was placed in a metal block heated to 50°C and the eluate was evaporated to dryness using nitrogen. The extract was taken up in 300 μl of 0.01 *M* sulphuric acid. The extract was mixed briefly using a vortex-mixer and 1 ml of hexane was added. The combined organic and aqueous layers were mixed for 30 a on a vortex-mixer and centrifuged at 2000 *g* for 5 min. The aqueous phase was transferred to a pre-treated 300-μl vial insert by inserting a Pasteur pipette through the organic layer and withdrawing the aqueous fraction. This extract was ready for analysis by HPLC.

Chromatography

The HPLC system was isocratic and consisted of an LKB 2150 pump, a Waters WISP 710B autoinjector, an in-line 0.2-μm filter, a Hypersil SAS C_1 guard column and a 25-cm Hypersil SAS C_1 (5 μm) analytical column. The flow-rate was maintained at 2.0 ml/min.

The electrochemical detector used was the ESA Model 5100A Coulochem detector with the Model 5010 analytical cell and the Model 5020 guard cell (supplied in the UK by Severn Analytical). The guard cell may be regarded as an accessory and was used to condition the mobile phase.

Cyclic voltammetry

Hydrodynamic voltammograms were constructed for each of the analytes. The first electrode of the analytical cell was held at 0.0 V and the potential at the second electrode was increased from 0.0 V in increments of 0.05 V. An injection of mixed tranquiliser standard was made onto the HPLC system after each adjustment. No response was seen up to +0.4 V. Peaks were seen above this potential at the retention times associated with each of the tranquiliser standards. No further significant response was observed after +0.7 V. The first electrode was then switched to +0.4 V and the second electrode increased from +0.4 V in steps of 0.05 V. The response of each analyte was measured at each potential. A hydrodynamic voltammogram is plotted in Fig. 1.

Operation

The guard cell was placed after the HPLC pump and before the injector. A potential of +0.75 V was applied to it in order to eliminate any response in the analytical cell which may be due to impurities in the mobile phase. The first electrode of the analytical cell was held at +0.4 V. The second electrode, from which the output was monitored, was set to +0.7 V. Using the combination of available electrodes in this manner is termed the sceen mode. The electrode 1 potential is at the foot of the current–voltage (*I–V*) curve for the analytes of interest. This is done to decrease background currents and to prevent unwanted peaks that result from eluents that oxidise at lower potentials than the analytes. The electrode 2 potential is set on the *I–V* curve plateau of the analyte with the highest oxidising potential.

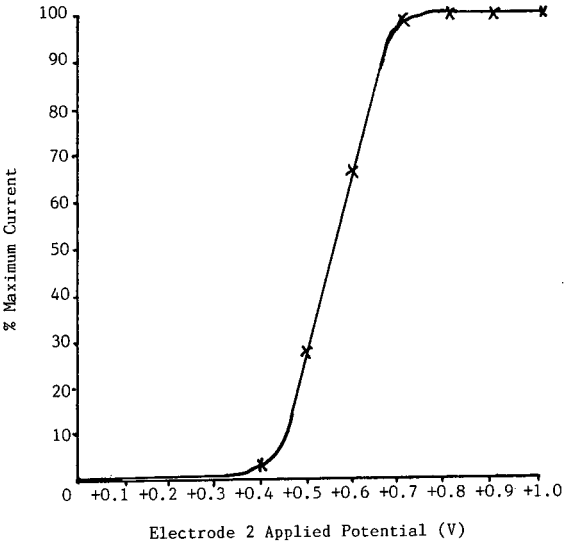

Fig. 1. Hydrodynamic voltammogram for tranquilisers. Electrode 1 was held at +0.4 V.

Compounds which oxidise at even higher potentials will not produce chromatographic peaks. This mode of operation increases the selectivity of the electrochemical detector.

Mobile phase was recirculated in the HPLC system by placing the waste outlet tubing into the reservoir. Recirculation is beneficial from both economic and environmental aspects, and has no detrimental effects when using this method. The mobile phase was regularly changed, especially if an increase in background current was observed in the analytical cell. Mobile phase was filtered through a 0.2-μm filter prior to use since the presence of small particles may block the porous graphite electrodes. An in-line filter was incorporated into the HPLC system after the injector as further protection for the electrodes. Sample extracts were filtered through 0.2-μm filters prior to analysis.

When an increase in back-pressure of more than 50 bar due to the analytical cell was noticed, it was isolated from the system and pumped through with 6 *M* nitric acid followed by water in order to clean it.

Validation protocol

Samples were processed in batches of eight. The spiking concentration and numbers of batches

TABLE I

RECOVERIES FROM PIGS' KIDNEY SPIKED WITH A MIXTURE OF COMPOUNDS AT 2 μg/kg

Values in parentheses are R.S.D.s (%).

Compound	Recovery (mean ± S.D.) (%)		
	Day 1 ($n=6$)	Day 2 ($n=6$)	Overall ($n=12$)
Azaperol	75 ± 2.2 (3.0)	81 ± 3.9 (4.9)	78 ± 4.1 (5.3)
Azaperone	74 ± 3.4 (4.6)	86 ± 5.0 (5.9)	80 ± 7.3 (9.1)
Carazolol	76 ± 4.1 (5.4)	70 ± 6.7 (9.5)	73 ± 6.2 (8.4)
Xylazine	68 ± 6.2 (9.3)	56 ± 3.4 (6.0)	62 ± 7.8 (12.5)
Haloperidol	69 ± 3.2 (4.7)	74 ± 2.2 (3.0)	72 ± 3.8 (5.3)
Acepromazine	70 ± 4.5 (6.3)	87 ± 4.8 (5.6)	78 ± 9.5 (12.2)
Propriopromazine	70 ± 4.5 (6.3)	88 ± 4.8 (10.7)	78 ± 9.5 (17.3)
Chlorpromazine	82 ± 2.7 (3.3)	95 ± 7.4 (7.8)	88 ± 8.8 (10.0)

TABLE II

RECOVERIES FROM PIGS' KIDNEY SPIKED WITH A MIXTURE OF COMPOUNDS AT 10 μg/kg

Values in parentheses are R.S.D.s (%)

Compound	Recovery (mean ± S.D.) (%)			
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Overall ($n=18$)
Azaperol	71 ± 4.3 (6)	80 ± 2.4 (3)	89 ± 1.9 (2)	80 ± 8.0 (10)
Azaperone	68 ± 4.2 (6)	76 ± 1.0 (4)	90 ± 5.0 (6)	78 ± 10.1 (13)
Carazolol	83 ± 6 (7)	66 ± 2.9 (4)	112 ± 14.1 (13)	87 ± 21.3 (24)
Xylazine	60 ± 3.7 (6)	64 ± 1.7 (3)	95 ± 8.9 (9)	73 ± 16.9 (23)
Haloperidol	68 ± 2.2 (3)	64 ± 2.0 (3)	94 ± 7.2 (8)	75 ± 14.1 (19)
Acepromazine	77 ± 2.3 (3)	83 ± 4.4 (5)	101 ± 6.5 (7)	87 ± 11.4 (13)
Propriopromazine	86 ± 3.8 (4)	83 ± 5.0 (6)	94 ± 6.5 (7)	88 ± 6.5 (7)
Chlorpromazine	81 ± 5.7 (7)	83 ± 8.5 (10)	100 ± 16.8 (17)	88 ± 13.9 (16)

TABLE III

RECOVERIES FROM PIGS' LIVER SPIKED WITH A MIXTURE OF STANDARDS AT 5 μg/kg

Values in parentheses are R.S.D.s (%).

Compound	Recovery (mean ± S.D.) (%)			
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Overall ($n=18$)
Azaperol	86 ± 3.6 (4.2)	91 ± 0.0 (0.0)	62 ± 2.8 (4.5)	80 ± 13.0 (16.4)
Azaperone	72 ± 6.2 (8.7)	77 ± 4.1 (5.3)	52 ± 0.8 (1.6)	67 ± 12.0 (17.9)
Carazolol	95 ± 3.2 (3.4)	89 ± 3.4 (3.9)	72 ± 3.9 (5.4)	85 ± 10.7 (12.6)
Xylazine	73 ± 15.2 (2.0)	78 ± 8.7 (11.2)	46 ± 2.5 (5.4)	66 ± 15.2 (23.1)
Haloperidol	76 ± 2.2 (2.9)	80 ± 11.6 (14.5)	50 ± 4.9 (9.8)	69 ± 15.2 (22.2)
Acepromazine	79 ± 2.5 (3.2)	81 ± 6.0 (7.4)	58 ± 6.1 (10.6)	72 ± 11.9 (16.5)
Propriopromazine	80 ± 3.1 (3.8)	79 ± 5.5 (7.0)	61 ± 1.3 (2.1)	74 ± 9.8 (13.3)
Chlorpromazine	91 ± 2.5 (2.7)	81 ± 5.3 (6.5)	64 ± 3.1 (4.8)	79 ± 12.0 (15.2)

TABLE IV

RECOVERIES FROM PIGS' LIVER SPIKED WITH A MIXTURE OF STANDARDS AT 25 μg/kg

Values in parentheses are R.S.D.s (%).

Compound	Recovery (mean ± S.D.) (%)			
	Day 1 ($n=6$)	Day 2 ($n=6$)	Day 3 ($n=6$)	Overall ($n=18$)
Azaperol	81 ± 4.6 (5.7)	82 ± 1.4 (1.7)	90 ± 1.6 (1.8)	84 ± 4.8 (5.7)
Azaperone	78 ± 3.9 (5.1)	80 ± 0.5 (0.6)	85 ± 1.6 (1.9)	81 ± 3.9 (4.8)
Carazolol	78 ± 6.6 (8.4)	84 ± 12.3 (14.7)	82 ± 4.2 (5.1)	81 ± 8.2 (10.2)
Xylazine	70 ± 3.9 (5.6)	70 ± 5.6 (8.0)	82 ± 3.2 (3.9)	74 ± 7.1 (9.6)
Haloperidol	67 ± 2.8 (4.2)	75 ± 1.6 (2.1)	84 ± 3.1 (3.6)	75 ± 7.7 (10.2)
Acepromazine	82 ± 5.3 (6.5)	90 ± 1.5 (1.6)	88 ± 3.4 (3.8)	87 ± 5.2 (5.9)
Propriopromazine	82 ± 4.4 (5.4)	92 ± 1.2 (1.3)	95 ± 2.4 (2.5)	90 ± 6.5 (7.2)
Chlorpromazine	82 ± 3.4 (4.2)	95 ± 1.6 (1.7)	94 ± 1.6 (1.8)	90 ± 6.7 (7.4)

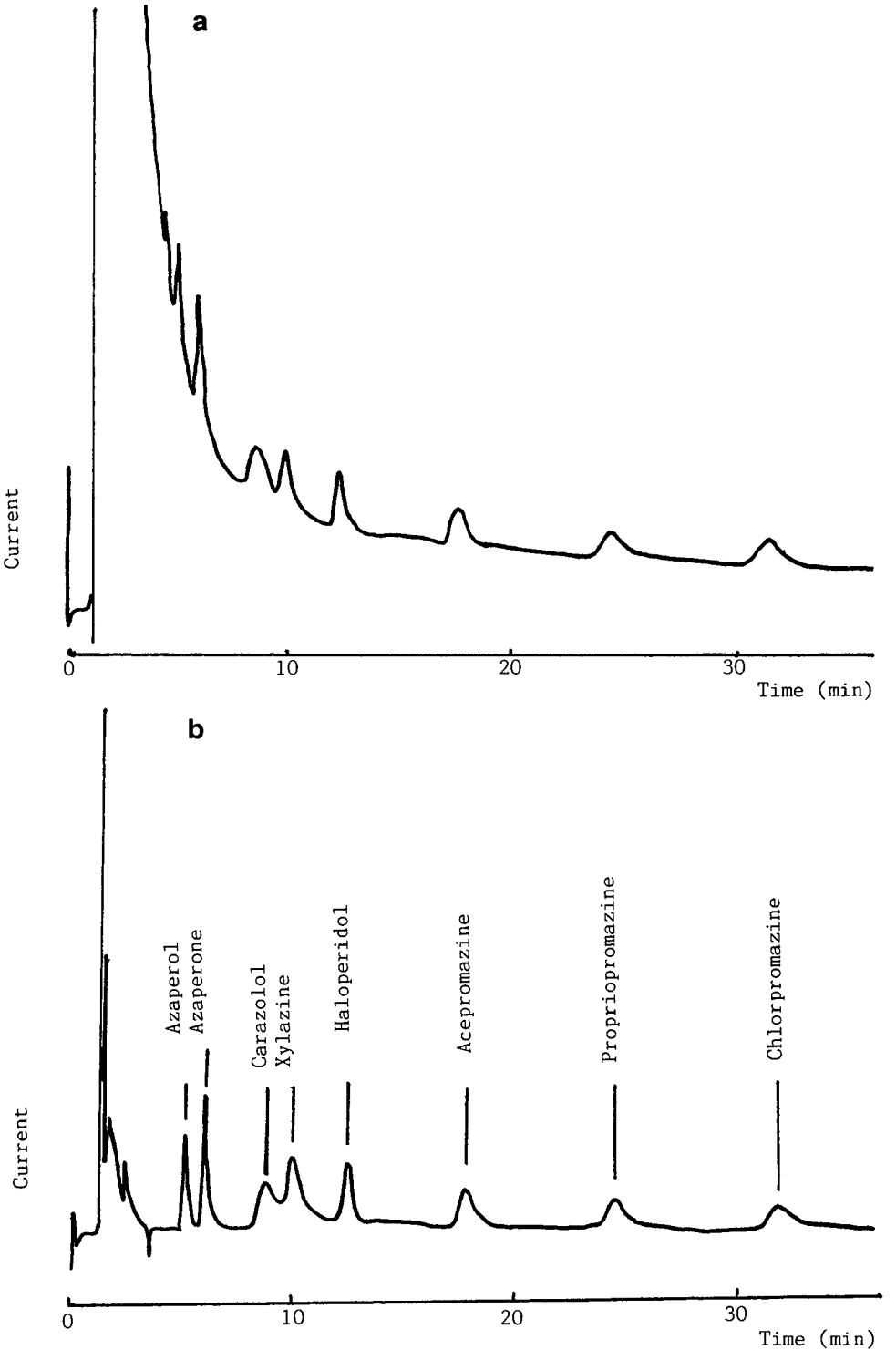

Fig. 2.

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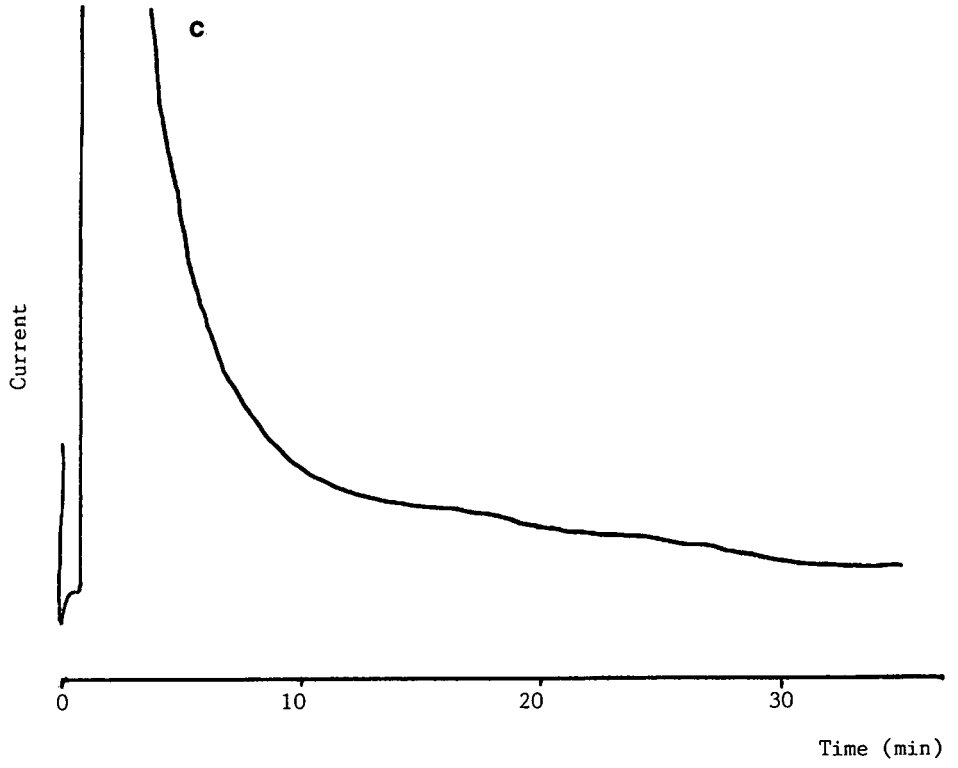

Fig. 2. ED chromatogram of (a) spiked tissue extract at 2 μg/kg, (b) mixed standard equivalent to 2 μg/kg and (c) blank tissue extract.

processed for each tissue type and concentration are detailed in Tables I–IV.

RESULTS AND DISCUSSION

It was found that each of the tranquilisers gave a strong electrochemical response, and that each compound was oxidised at a similar potential (+0.4 to +0.7 V). This ED method has been adopted as a standard operating procedure in our laboratory and is routinely applied to the analysis of tranquilisers in meat samples.

The mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) for the recovery of each standard in both tissue types are summarised in Tables I–IV. Typical chromatograms for a standard, spiked tissue extract and a blank tissue extract are shown in Fig. 2.

Limits of determination were found to be ten times lower than those found using UV detection.

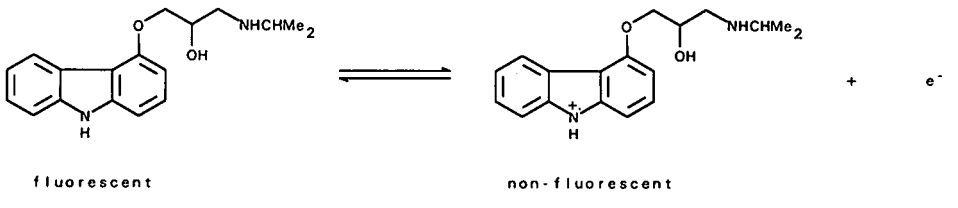

Fig. 3. Proposed reaction for the oxidation of carazolol. Me = Methyl.

TABLE V

COMPARISON OF LOWEST VALIDATION LIMITS FOR KIDNEY USING UV, FLUORESCENCE AND ELECTROCHEMICAL DETECTION

Compound	Lowest validation limit (μg/kg)		
	UV	Fluorescence	ED
Azaperone	20	10	2
Azaperol	20	10	2
Carazolol	20	5	2
Xylazine	20	N.A.[a]	2
Haloperidol	20	N.A.	2
Acepromazine	20	N.A.	2
Propriopromazine	20	N.A.	2
Chlorpromazine	20	N.A.	2

[a] N.A. = Fluorescence detection not possible.

Significant improvements were also found for those compounds normally measured using fluorescence detection.

The electrochemical detector may be used in combination with other detectors. The peak associated with carazolol is not seen by fluorescence if the electrochemical detector is in series in front of the fluorescence detector. The peak is visible, however, if the detector positions are reversed. This suggests that the species produced when carazolol is oxidised is no longer fluorescent under the same conditions. This fact may be used to assist confirmation of samples screened as positive for carazolol, by analysing an extract twice with the detector positions reversed, or by using two detectors, one in front and one behind the electrochemical detector (Fig. 3).

CONCLUSIONS

ED improves selectivity and enables the determiantion of tranquiliser residues in pig liver in addition to kidney samples. The method for the determination of tranquiliser residues presented in this paper offers up to ten-fold improvements in limits of determination when compared to existing methods (Table V).

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Determination of indolic compounds in pig back fat by solid-phase extraction and gradient high-performance liquid chromatography with special emphasis on the boar taint compound skatole

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ABSTRACT

A gradient high-performance liquid chromatographic method for the separation of thirteen indolic compounds and the determination of seven of them was developed. The indolic compounds include indole and skatole, both of which are involved in the boar taint. A solid-phase extraction method for sample preparation for the determination of the indolic compounds in pig back fat is described. The indolic compounds are extracted with acetone–Tris buffer, and lipids and fatty acids are trapped on a Bond-Elut C_{18} column. Fluorescence (excitation and emission at 280 and 340 nm, respectively) is used for selective detection. The detection limit for indole and skatole is 15 $\mu g\ kg^{-1}$ back fat. The sample preparation procedure is simple and the method is sensitive and reproducible. The method was compared with a spectrophotometric method for the determination of skatole in back fat.

INTRODUCTION

In many countries, castration of male pigs has been practised for centuries in order to avoid the occurrence of male odour (boar taint) in meat from 5–10% of the animals. The male odour is only a problem when meat or meat products are heated by the consumer prior to consumption. However, the advantages of entire male, *i.e.* uncastrated, pig production compared with hog production are large; the animal welfare aspect is important, male pigs grow faster, they suffer less illness and the meat is leaner with a better dietary composition of fatty acids.

The compounds responsible for the boar taint are known to be skatole [1–3] and the pheromone 5α-androst-16-en-3-one [4]. Other steroids that have also been associated with the boar taint are 5α-androst-16-en-3α-ol and 5α-androst-16-en-3β-ol [5–8].

Several methods for the determination of skatole have been described; these include UV spectrophotometric, high-performance liquid chromatographic (HPLC), thin-layer chromatographic (TLC) and gas chromatographic (GC) procedures. For the determination of skatole in gastrointestinal content, TLC [9], HPLC with UV detection [10] and GC [11] methods have been described. However, the sample preparation procedures used are not suitable for the determination of skatole in adipose tissue.

Different chromatographic methods for the determination of skatole in back fat using normal-phase [12] and reversed-phase HPLC [13,14] or GC [15,16] have been described. The critical step in the determination of skatole in adipose tissue is the sample preparation procedure, because skatole is a very lipophilic compound and the chromatographic system is disturbed or destroyed by fat. Sample preparation procedures used include liquid–liquid extraction [16], steam distillation [3] and solid-phase extraction in the normal-phase [12,15] and reversed-phase modes [14].

A routine UV method involves extraction of fat samples with Tris–acetone followed by measurement of the absorbance at 580 nm after derivatiza-

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tion with 4-dimethylamino benzaldehyde [17]. This method, which when fully automated has a capacity of 200 samples per hour, is currently used for analysing large numbers of carcasses on slaughterhouse lines in order to sort out carcasses with unacceptable male odour. The method, however, is believed to be unspecific, because of interference from other indolic compounds. Nevertheless, there is an excellent correlation between the results of this method and the judgement by a trained taste panel [18].

The factors causing the occurrence of male odour are mainly unknown. In order to elucidate the mechanisms behind the boar taint problem, investigations are in progress to study the physiological, microbiological, hereditary and practical problems responsible for boar taint.

In order to have a general method for the determination of skatole and indole and also several other indolic compounds which may be precursors or, alternatively, metabolites of skatole, two gradient HPLC methods were developed. For the determination of indolic compounds in back fat samples, a solid-phase extraction procedure for the removal of fat from the samples was developed. The solid-phase extraction is not used in the traditional manner, *i.e.*, concentration of compounds followed by selective elution of interfering compounds and finally elution of the compounds of interest. In the proposed method, solid-phase extraction is used for trapping lipids and fatty acids while the indolic compounds pass through the column. The extract is analysed directly, which makes sample preparation simple and fast. The method developed is compared with the spectrophotometric method for the determination of skatole described by Mortensen and Sørensen [17].

EXPERIMENTAL

Chemicals

Indole, 3-indoleacetic acid (IAA), 3-indolyacetonitrile (IACN), 3-indolebutyric acid, 2-indolecarboxylic acid (2-ICA), 3-indolecarboxylic acid (3-ICA), 3-indoleethanol (IEtOH), 3-indolemethanol (IMeOH), 2-methylindole (2-MID), skatole (3-methylindole), tryptamine and tryptophan (THY) were obtained from Sigma (St. Louis, MO, USA). Demineralized water was treated in a Milli-Q Plus water purification system from Millipore (Bedford, MA, USA). Methanol, acetone, acetonitrile (ACN) and tetrahydrofuran (THF) were of HPLC grade from Romi (Loughborough, UK). All other chemicals were of analytical-reagent grade.

Back fat samples

Back fat samples for validation of the method were obtained from a local butcher. Prior to use, they were tested for content of indolic compounds to ensure a low content of skatole. Back fat samples used in the developmental stages were selected at the local abattoir based on a screening of the skatole content of the carcasses by means of the method described by Mortensen and Sørensen [17].

Conditioning of solid-phase extraction columns

A Bond-Elut column (C_{18}, 500 mg, 6 ml, from Analytichem, Harbor City, CA, USA) was conditioned prior to use by passing two 5-ml aliquots of methanol, acetone and acetone/0.1 *M* Tris–HCl buffer (pH 7.5)–0.1 *M* sodium sulphite (acetone–Tris) (75:25:1, v/v/v) through the column by means of a Vac Elute SPS 24 (Analytichem). Prior to application of tissue homogenate, the column was cooled by slowly passing 5 ml of ice-cold acetone–Tris through the column.

High-performance liquid chromatography

HPLC system 1 was a Hitachi system from Merck (Darmstadt, Germany) consisting of a Model 655 A-40 autosampler, a Model L-6200 gradient pump, a Model F-1000 fluorescence detector and Model D-6000 HPLC manager software installed in a Compaq Deskpro 386s PC.

The column was a LiChrospher RP-Select B (5 μm) (250 mm × 4 mm I.D.) fitted with a 4-mm RP-Select B precolumn (LiChroCART system, Merck). The mobile phases consisted of (A) ACN–50 m*M* potassium phosphate buffer (pH 6.0) (5:95, v/v) and (B) ACN–water (90:10, v/v) with the following gradient profile: 0–16.0 min, 0–80% B; 16.0–16.1 min, 80–100% B; 16.1–18.0 min, 100% B; 18.0–18.5 min, 100–0% B; 18.5–21.0 min, 0% B. The flow-rate was 1.2 ml min^{-1}.

HPLC system 2 was an LC-Analyst system from Perkin-Elmer (Norwalk, CT, USA) consisting of a Model 620 four-solvent pump with continuous helium degassing, an ISS 200 autosampler fitted with a 150-μl loop, an LC-235 diode-array detector, an

LC-240 luminescence detector, a DEC station 316 SX PC, an Analyst software kit, a PESOS software kit and an ML HPLC column oven (Mikrolab, Aarhus, Denmark). A PEEK back-pressure regulator on 40 p.s.si. (Upchurch Scientific, Oak Harbor, WA, USA) was installed after the detectors.

The column was a Superspher RP-8 (4 μm) (120 mm × 4 mm I.D.) operated at 40°C, fitted with a 4-mm RP-8 precolumn LiChroCART system, Merck). The composition of the mobile phases was (A) methanol–acetic acid–water H_2O (5:2:93, v/v/v); (B) THF–water (90:10, v/v) with the following gradient profile: equilibration, 5 min 15% B; 0–11.5 min, 15–47% B; 11.5–11.7 min, 47–70% B; 11.7–13.9 min, 70% B; 13.9–14.2 min, 70–15% B; 14.2–15.2 min, 15% B. The flow-rate was 1.2 ml min^{-1}.

The principle of detection in both systems was fluorescence with excitation at 285 nm and emission at 340 nm. In all assays 40 μl of the sample were injected. 2-MID was used as an internal standard for the determination of indole and skatole and 2-ICA for the determination of THY, IAA, IACN, IPA and IEtOH. The internal standards were added to the samples prior to sample pretreatment to a final concentration of 0.1 mg l^{-1}.

The linearity of the calibration graphs based on peak area was investigated in the range 0.0001– 5.0 mg l^{-1}.

Stability of the indolic compounds

The stability of skatole, indole, IAA, IACN, IEtOH, IPA, 2-MID, 2-ICA and THY was determined in the following different solutions at ambient temperature. (1) 2.5% trichloroacetic acid; (2) acetone–0.1 *M* Tris–HCl buffer (pH 7.5)–0.1 *M* sodium sulphite (75:25:1; v/v/v); and (3) acetone–0.1 *M* Tris–HCl buffer (pH 7.5) (75:25; v/v).

Sample pretreatment

A 5.00-ml volume of acetone–Tris and 50 μl of an internal standard solution containing 10 mg l^{-1} of 2-MID and 2-ICA were added to 2.50 g of back fat. After homogenization by means of a Kinematica (Littau, Switzerland) Polytron PT 3000 fitted with a 20-mm aggregate, the samples were sonicated for 5 min and cooled in an ice-bath for 15 min. The ice-cold solution was then passed through an activated and chilled Bond-Elut column. The column was washed by passing two 1.0-ml portions of ice-cold acetone–Tris through it. The eluate was diluted with three parts of water and analysed.

Gravimetric determination of extractable matter removed by solid-phase extraction

A 10.0-g amount of back fat was homogenized in 200.0 ml of acetone–Tris, cooled on ice and filtered. First, three 20.0-ml aliquots of the filtrate were evaporated to dryness. Next, three 20.0-ml aliquots of the filtrate were passed through activated Bond-Elut columns and were subsequently evaporated to dryness. Finally, three 20.0-ml acetone–Tris aliquots were evaporated to dryness; all were further dried for 1 h at 110°C. The residues from evaporation were analysed by weighing.

Effect of sonication on recovery

The recoveries from samples spiked with 0.2 mg/kg of indole, IAA, THY and skatole were determined after sonication for 0, 5, or 30 min prior to solid-phase extraction.

Validation

The recovery and the intra- and inter-assay variability of the sample preparation procedure were determined by spiking three different back fat samples with a low content of indolic compounds at three different levels (see Table I).

Application

Samples (137) of back fat from uncastrated male pigs were analysed in duplicate using HPLC system 1. The same samples were also analysed by a spectrophotometric method [17,19] and the results were compared.

RESULTS AND DISCUSSION

Selectivity of the chromatographic system

As part of the boar taint project, it was decided to develop at least one general-purpose HPLC system for the determination of indolic compounds which could be precursors to skatole or indole formed in the gastrointestinal tract by various bacteria (THY, IAA, IEtOH, IPA and tryptamine) [20]. Other possible metabolites of skatole (IMeOH and 3-ICA) were included. IAA may be a precursor to skatole, as heating causes decarboxylation above 160°C. It was intended to use 2-ICA and 2-MID as internal

TABLE I

SPIKING LEVELS OF INDOLIC COMPOUNDS ADDED TO SAMPLES ORIGINALLY CONTAINING LOW AMOUNTS OF INDOLE AND SKATOLE IN ORDER TO DETERMINE RECOVERIES AND WITHIN-DAY AND BETWEEN-DAY COEFFICIENTS OF VARIATION

Level	Concentration added (mg kg^{-1})						
	THY	IAA	IRO	IEtOH	IACN	Indole	Skatole
1	0.5	0.02	0.02	0.02	0.02	0.05	0.05
2	2.0	0.08	0.08	0.08	0.08	0.2	0.2
3	5.0	0.2	0.2	0.2	0.2	0.5	0.5

standards. A LiChroCART Select B column was selected for development of one of the systems because of poor peak shape of tryptamine on most other stationary phases investigated. The good selectivity of the system is shown in Fig. 1. The total time of analysis is 25 min for thirteen indolic compounds.

Inspecting the chromatogram of standards in Fig. 1a, a total of fourteen peaks can be counted. This is due to the fact that IMeOH was found to be highly unstable under the conditions applied. After preparation of a fresh solution of IMeOH in methanol–water (1:1, v/v) and repeated analysis as a function of time, it was discovered that the original peak slowly split into two with the emerging peak appearing later in the chromatogram. The identity of the compound in this peak is not known.

In order to develop a faster alternative system with a selectivity different from the acetonitrile system, a system using a Superspher RP-8 column (4 μm) and THF, methanol and acetic acid in the mobile phase was developed. From Fig. 2 it is seen that the selectivity of the latter system is excellent and, just as important, different from the first. This system cannot be used for the determination of tryptamine and IMeOH because of the poor peak shapes of these compounds. Efforts to decrease the time of analysis by increasing the content of organic modifiers under the initial conditions were not successful, because k' for THY remained constant when increasing the content of THF in the mobile phase (Fig. 3).

Principle of detection

Both UV and fluorescence detection were investigated. Fluorescence detection was selected because of the better selectivity in the detection of indolic

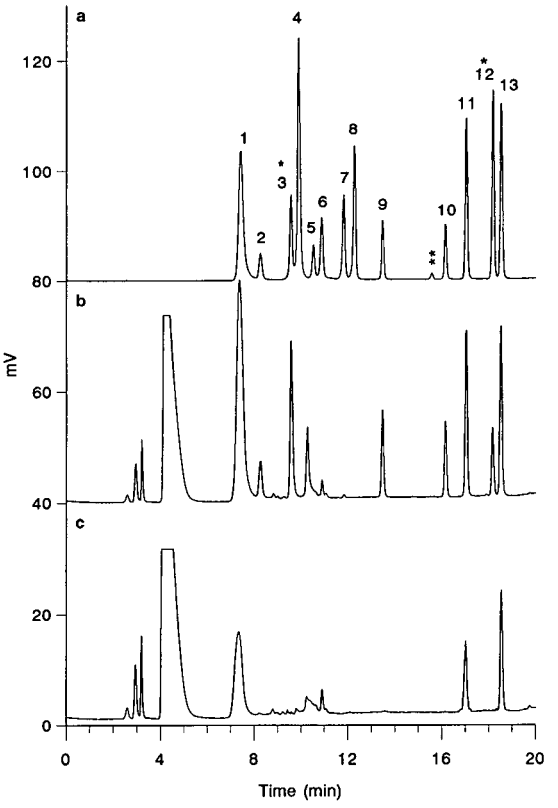

Fig. 1. Selectivity of the chromatographic system 1. Peaks: 1 = tryptophan; 2 = indoleacetic acid; 3 = 2-indolecarboxylic acid; 4 = 3-indolecarboxylic acid; 5 = tryptamine; 6 = 3-indolepropionic acid; 7 = 3-indolemethanol; 8 = 3-indolebutyric acid; 9 = 3-indoleethanol; 10 = 3-indoleacetonitrile; 11 = indole; 12 = 2-methylindole; 13 = skatole; * = internal standards; ** = rearrangement product of 3-indolemethanol. Column: LiChroCART Slect B (5 μm; 250 mm × 4 mm I.D.). The mobile phases consisted of (A) ACN–50 m*M* potassium phosphate buffer (pH 6.0) (5:95, v/v) and (B) ACN–water (90:10, v/v) with a gradient profile from 0 to 80% B in 16.0 min. Flow-rate: 1.2 ml min^{-1}. (c) Back fat sample with a content of 0.12 and 0.19 μg g^{-1} of indole and skatole, respectively; (b) same sample spiked with nine different indolic compounds; (a) standard solution containing 0.5 μg ml^{-1}.

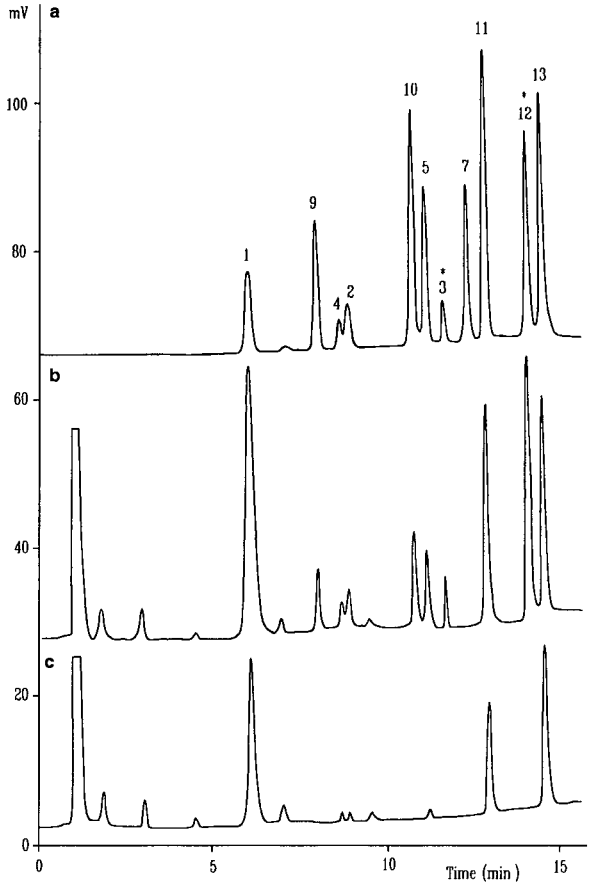

Fig. 2. Selectivity in chromatographic system 2. Identity of the compounds as in Fig. 1. Column: Superspher RP-8 (4 μm, 120 mm × 4 mm I.D.), operated at 40°C. The mobile phases consisted of (A) methanol–acetic acid–water (5:2:93, v/v/v) and (B) THF–water (90:10, v/v) with a gradient profile from 15 to 47% B in 11.5 min. Flow-rate: 1.2 ml min^{-1}. (c) Back fat sample with a content of 0.16 and 0.19 $\mu g\ g^{-1}$ of indole and skatole, respectively; (b) same sample spiked with eleven different indolic compounds; (a) standard solution containing 0.5 $\mu g\ ml^{-1}$.

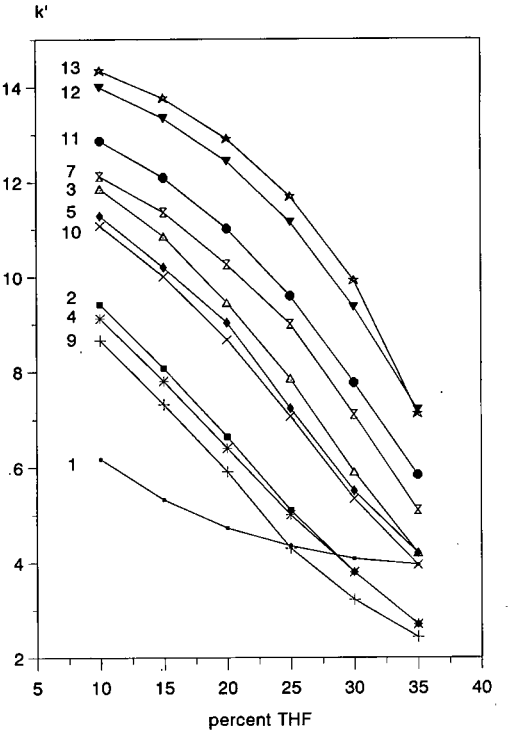

Fig. 3. Values of k' for the different indolic compounds as a function of the concentration of THF in the initial mobile phase of the gradient system (system 2). Identity of compounds as in Fig. 1.

compounds and an at least ten times better sensitivity.

Linearity, detection and quantification limit

The detection and quantification limits were determined for the different indolic compounds (Table II). The detection limits (signal-to-noise ratio = 3) were determined in standard solutions. The quantification limits were defined as ten times the detection limit unless authentic samples showed less sensitivity. The quantification limit of THY could not be verified because it proved impossible to find a sample with low content of THY. For IPA the detection limit is fairly high, as a system peak close to the IPA peak makes the quantification of IPA difficult at low concentrations. A quantification limit of 15 $\mu g\ kg^{-1}$ for skatole is satisfactory when the determinations are used for elucidations of relationships between smell or taste and content. The threshold for sensing skatole is 0.2 mg kg^{-1} [18].

Indolic compounds identified in pig back fat

During the development of the chromatographic systems, some back fat samples were analysed for their content of various indolic compounds in order to verify that the selectivity of the method was sufficient for determination of THY, IAA, indole and skatole. During these investigations the presence of IMeOH, IPA, IACN and IEtOH was discovered. Their presence was corroborated by spiking with

TABLE II

DETECTION AND QUANTIFICATION LIMITS FOR THE DIFFERENT INDOLIC COMPOUNDS IN HPLC SYSTEM 1

The detection limits (signal-to-noise ratio = 3) were determined using standard solutions. The quantification limits were defined as ten times the detection limit unless authentic samples showed less sensitivity.

Parameter	THY	IAA	IPA	IEtOH	IACN	Indole	Skatole
Detection limit ($\mu g\ kg^{-1}$)	4.0	2.0	0.8	0.4	0.4	0.2	0.2
Quantification limit ($\mu g\ kg^{-1}$)	40	20	100	15	15	15	15
Linearity ($\mu g\ kg^{-1}$)	10–9000	4–1500	8–1500	4–1500	4–1500	2–1500	2–1500

known amounts of standards in the two systems; for IMeOH, however, only in system 1, owing to the poor chromatographic properties of IMeOH in the acidic system 2. In order to determine if the presence of IPA, IACN and IEtOH is general for back fat, the method was validated for the determination of these compounds. IMeOH was not included in the validation owing to its instability.

Choice of internal standards

During development of the chromatographic systems, several not naturally occurring indolic compounds were considered as possible internal standards in the systems. Using HPLC system 2 and 2-ICA as internal standard, the coefficients of variation (C.V.s) for the determination of THY, IAA, skatole and indole were 0.5, 1.2, 10.4 and 11.6%, respectively, whereas the C.V.s when using 2-MID were 11.0, 11.2, 0.8 and 1.7%, respectively. Hence 2-ICA is suitable as internal standard for the more polar idolic compounds such as THY and IAA, whereas 2-MID is suitable for the more lipophilic indolic compounds such as indole and skatole. This is in good agreement with general practice for the selection of internal standards.

Stability of the indolic compounds

A solution of skatole in acetone–Tris solutions, with and without addition of sodium sulphite, is stable for at least 60 h (less than 2% decrease in peak area). However, in a 2.5% trichloroacetic acid solution skatole is stable for only 10 h (less than 2% decrease in peak area). The pattern is similar for the other indolic compounds. The stability of IMeOH was not tested separately. As a consequence of observations made during the development of the chromatographic system, it was discovered that a standard solution of IMeOH in methanol–water (1:1, v/v) is highly unstable. During a few hours the peak split into two different peaks (Fig. 1).

Conditioning of the columns

Chilling of the solid-phase extraction columns prior to application of tissue homogenate and also the application of cold homogenate were found to be essential. If the homogenate was at ambient temperature the lipids would melt, with the risk of liquid lipids passing through the columns, and consequently lipids would later be injected on to the analytical column.

Effect of sonication on recovery

The duration of the sonication had no significant effect on the recovery, but sonication for 5 min was maintained in order to ensure that equilibrium of the internal standards between the fat particles and the solvent was achieved.

Removal of fat by solid-phase extraction

Several procedures for removal of fat from the samples were investigated. A very simple procedure using microwave melting followed by extraction of the melted fat with methanol [21] was initially investigated. The method was not adopted due to a rapid increase in the column back-pressure and difficulties with proper assessment of the recovery of this procedure. An obvious sample preparation procedure for removal of fat would be solid-phase extraction using normal-phase conditions as in the method described by García-Regueiro *et al.* [12], who used Florisil columns. The method was tried but was unsatisfactory due to problems with some compounds (IAA, indole and skatole) subliming away during evaporation of the sample solutions.

Normally solid-phase extraction systems are used for the concentration of compounds by retaining them on the column, followed by elution of interfering compounds with a suitable eluent and finally elution of the compounds of interest. In the present method the compounds of interest are not retained by the column, but the interfering fat is retained by the stationary C_{18} phase.

The effectiveness of the procedure in removing fat from the samples was assessed gravimetrically. The average residue of evaporation of pure extract was 96.8 ± 1.8 mg, that of evaporation of extract after passage through a Bond-Elut column was 91.5 ± 0.50 mg and that of evaporation of acetone–Tris was 86.5 ± 2.2 mg. Hence the solid-phase extraction procedure is capable of removing 5.3 mg, corresponding to 51% of the extractable matter. The rest of the extractable compounds can pass through the analytical column, a fact demonstrated by no significant increase in column back-pressure and no change in column selectivity during the *ca.* 1000 sample analyses to date.

The eluent/extraction medium contains 75% acetone, which makes it a very strong eluent compared with the initital conditions of the HPLC systems; the samples have to be diluted with three parts of water in order not to disturb the peak shape of the first-eluting peaks.

Validation

It was not possible to find back fat samples for validation that did not contain any of the indolic compounds. THY, indole and skatole were always present. As regards to indole and skatole, however, it was easy to find samples with a low content.

Only HPLC system 1 was validated. The recovery of the sample preparation procedure was assessed by spiking samples with a low content of indolic compounds with known amounts of the same indolic compounds. The levels used for spiking (Table I) were chosen after an investigation of the content of indolic compounds in about twenty back fat samples. The recovery was calculated from the difference between the average value recorded during 5 days for spiked an unspiked samples.

The average recoveries for skatole and indole are 92% and 117%, respectively, when using 2-MID as internal standard. The recoveries of the two compounds are different but stable. An explanation for this difference may be that the extraction is not 100% partly because the samples are only extracted once and the three compounds have different lipophilicities, a fact which also is reflected in the order of elution in the chromatograms. For the other compounds (except IPA) the recoveries are stable only they vary slightly at level 1 (IAA, IACN and IEtOH) because this level is close to the quantification limit.

The within-day ($n=7$) and between-day ($n=5$) C.V.s for the different indolic compounds are given in Table III. For THY, indole and skatole the C.V.s are acceptable at all levels used for the validation. For the other four indolic compounds the C.V.s are fairly high at the lowest concentration because the concentration is just above the quantification limit of the system. The C.V. for IPA is generally high owing to problems with a system peak close to the IPA peak.

Hence HPLC system 1 can be used for the determination of THY, indole and skatole at all the levels investigated. The method can be used for the determination of concentrations of IAA, IEtOH and IACN higher than level 1, and for IPA at concentrations higher than level 2.

Application

Table IV summarizes the concentrations of the different indolic compounds determined using HPLC system 1. Only THY is present in all the samples whereas at least trace levels of skatole and indole are found in all samples. IPA, IEtOH and IACN are not present in significant amounts.

On comparing the content of skatole determined by HPLC and the spectrophotometric method (Fig. 4), a good correlation ($r = 0.973$) between the two methods is seen. The equation of the regression line is $y = 1.0743x - 0.0671$ (y = HPLC result; x = spectrophotometric result). Hence the spectrophotometric method tends to overestimate the content of skatole, when the concentration is low, compared with the HPLC method. This is probably due to the unspecificity of the spectrophotometric method. In order to establish whether some of the other indolic compounds determined by the HPLC method contribute to the results from the spectrophotometric method, the results were compared by means of the multivariate analysis program Unscrambler [22] (Camo, Trondheim, Norway). It was not pos-

TABLE III

RECOVERIES AND INTRA- AND INTER-ASSAY VARIABILITIES DETERMINED BY SPIKING THREE SAMPLES, A, B AND C, WHICH ORIGINALLY CONTAINED LOW AMOUNTS OF INDOLE AND SKATOLE WITH KNOWN AMOUNTS OF THE INDOLIC COMPOUNDS

The levels used for spiking are given in Table I. The recovery was calculated from the difference between the average recorded during 5 days for spiked and unspiked samples.

Compound	Level	Sample	Assay conc.: mean ± S.D. ($n=7$) (mg kg^{-1})	Recovery (%)	Coefficient of variation		Compound	Assay conc.: mean ± S.D. ($n=7$) (mg kg^{-1})	Recovery (%)	Coefficient of variation	
					Within day ($n=7$)	Between days ($n=5$)				Within day ($n=7$)	Between days ($n=5$)
Skatole	0	A	0.009 ± 0.001	–	7.8	12	Indole	0.009 ± 0.001	–	10.4	12.0
		B	0.021 ± 0.003	–	15.3	21.9		0.008 ± 0.003	–	46.9	10.7
		C	0.052 ± 0.004		8.1	10.3		0.006 ± 0.002	–	27.2	31.7
	1	A	0.054 ± 0.004	93.2	7.5	5.2		0.069 ± 0.004	119.3	5.3	6.3
		B	0.072 ± 0.003	95.9	4.4	8.6		0.069 ± 0.005	117.5	7.0	3.3
		C	0.106 ± 0.002	93.0	1.9	6.1		0.064 ± 0.002	113.9	3.1	3.6
	2	A	0.189 ± 0.004	90.6	2.2	2.0		0.234 ± 0.006	117.4	2.9	4.3
		B	0.211 ± 0.008	92.6	4.0	4.9		0.258 ± 0.011	118.8	4.2	5.0
		C	0.238 ± 0.008	90.7	3.7	6.0		0.228 ± 0.008	116.5	3.5	6.1
	3	A	0.452 ± 0.016	88.5	3.6	2.7		0.598 ± 0.038	117.5	6.4	2.7
		B	0.513 ± 0.022	92.8	4.5	5.5		0.671 ± 0.054	118.9	8.1	8.1
		C	0.514 ± 0.009	90.1	1.7	4.5		0.551 ± 0.016	113.5	2.9	5.5
IAA	0	A	–	–			IPA	–	–		
		B	–	–				–	–		
		C	–	–				–	–		
	1	A	0.015 ± 0.002	76.3	14.9	24.3		0.038 ± 0.003	132.3	7.8	50.4
		B	0.063 ± 0.011	75.0	18.8	21.9		0.025 ± 0.005	122.5	22.9	50.8
		C	0.021 ± 0.001	90.0	5.4	20.0		0.069 ± 0.006	214.0	8.8	28.8
	2	A	0.065 ± 0.003	83.8	5.6	6.8		0.085 ± 0.004	114.8	5.7	19.1
		B	0.052 ± 0.010	83.4	19.8	17.3		0.122 ± 0.045	100.0	37.3	15.4
		C	0.081 ± 0.004	93.8	5.2	4.2		0.126 ± 0.014	88.0	11.2	25.6
		B	0.170 –	85.0	–	5.8		0.217 ± 0.011	86.4	4.7	14.7
		C	0.202 ± 0.002	89.7	1.1	9.2		0.219 ± 0.001	94.0	0.5	16.6

IEtOH	0	A	–	–			IACN	–	–			
		B	–	–				–	–			
		C	–	–				–	–			
	1	A	0.025 ± 0.06	107.0	24.3	10.9		0.016 ± 0.001	86.9	9.3	6.4
		B	0.027 ± 0.003	104.1	12.8	15.8		0.021 ± 0.002	93.4	8.9	11.4
		C	0.021 ± 0.001	95.0	5.4	12.5		0.019 ± 0.001	90.8	6.0	13.5
	2	A	0.084 ± 0.007	97.5	9.1	5.2		0.068 ± 0.001	84.3	2.7	4.5
		B	0.091 ± 0.018	99.7	19.8	8.1		0.072 ± 0.008	87.6	11.4	5.0
		C	0.079 ± 0.004	94.7	5.2	7.1		0.074 ± 0.014	87.0	18.9	10.7
	3	A	0.183 ± 0.010	93.2	5.7	3.5		0.166 ± 0.009	83.2	5.8	6.1
		B	0.217 ± 0.015	97.2	7.0	7.7		0.165 ± 0.018	84.9	11.3	7.5
		C	0.203 ± 0.007	94.6	3.5	8.9		0.193 ± 0.007	85.5	3.6	12.1
THY	0	A	2.49 ± 0.03	–	1.2	4.5					
		B	2.93 ± 0.21	–	7.2	5.6					
		C	2.31 ± 0.22	–	9.6	2.8					
	1	A	3.01 ± 0.17	91.3	2.3	5.7					
		B	3.34 ± 0.07	94.8	5.3	9.0					
		C	2.73 ± 0.07	81.3	2.7	3.8					
	2	A	4.25 ± 0.07	92.3	1.8	3.7					
		B	4.89 ± 0.04	90.1	7.0	2.8					
		C	4.29 ± 0.03	92.0	0.8	4.6					
	3	A	7.23 ± 0.14	94.4	1.9	2.0					
		B	8.52 ± 0.43	95.8	5.1	4.3					
		C	7.24 ± 0.08	95.2	1.2	1.8					

TABLE IV

SUMMARY OF THE CONCENTRATIONS OF THE DIFFERENT INDOLIC COMPOUNDS DETERMINED BY MEANS OF HPLC SYSTEM 1

The samples used were not selected randomly, but based on the skatole content determined by means of a spectrophotometric method [17]. Hence the samples cannot be regarded as representative for the general level of skatole in Danish domestic pigs. The average level of skatole in Danish male pigs is currently 0.10 ppm (n > 100 000).

	Concentration (mg kg^{-1})					
	THY	IAA	IEtOH	Indole	Skatole	Skatole[a]
Highest	8.85	0.038	0.028	0.302	1.71	1.49
Lowest	1.28	<0.02	<0.015	<0.015	<0.015	0.05
Average	3.51	0.003	0	0.035	0.25	0.30

[a] Determined by means of the spectrophotometric method described by Mortensen and Sørensen [17].

sible to obtain a better correlation, hence it may be argued that none of the other indolic compounds contributes to the results obtained by the spectrophotometric method.

The samples used for the appplication were not randomly selected, but on the basis of the skatole content determined by means of the spectrophotometric method. Some of the samples were used for an investigation of the relationship between the content of indolic compounds and the taste or smell. Hence the samples cannot be regarded as representative of the general level of skatole and indole in Danish domestic pigs. The average level of skatole in male pigs is currently 0.10 ppm (n > 100 000).

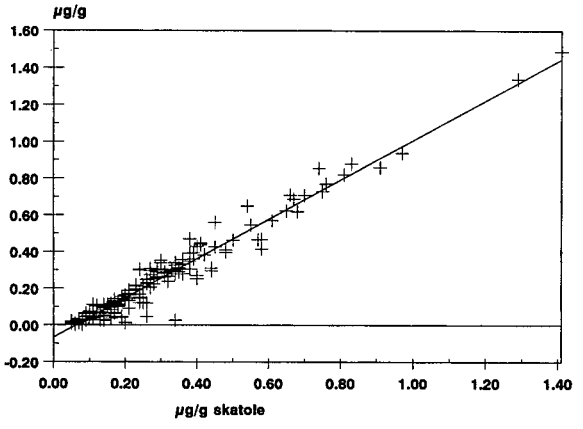

Fig. 4. Comparison of skatole determination in 137 pig back fat samples by use of the spectrophotometric method described by Mortensen and Sørensen [17] (y-axis) and the proposed HPLC method (x-axis). The correlation between the methods is 0.973 and the regression equation is $y = 1.0743x - 0.0671$.

Table V summarizes the present results and those reported by other workers. The results are not directly comparable, owing to differences in the way the samples were selected. The Spanish samples [13] were selected on the basis of the presence of boar taint (subjective judgement). The samples used in this study were selected on the basis of skatole determined by a spectrophotometric method [17]. Among the Danish samples was found the highest content of skatole, whereas the highest concentration of indole was reported in the German results [14].

CONCLUSIONS

As part of a project on studying the boar taint problem, a gradient HPLC method for the separation of thirteen indolic compounds was developed. The method has been validated for determination of seven indolic compounds in pig back fat. Only four of these compounds were found in significant amounts in 137 back fat samples analysed. The skatole concentrations obtained by the method were compared with those given by a spectrophotometric method for the determination of skatole; the correlation between the methods was excellent (r = 0.973).

When producing uncastrated male pigs, it is important to have a method capable of determining the concentration of skatole in the carcass. This method could be a HPLC procedure. The gradient

TABLE V

COMPARISON OF THE CONCENTRATIONS OF INDOLE AND SKATOLE OBTAINED BY THE PRESENT METHOD AND OTHER PUBLISHED METHODS

The other methods used were HPLC [13,14] and GC [15].

	Present method			García-Regueiro and Diaz [13]			Gibis *et al.* [14]			Porter *et al.* [15]	
	Indole (mg kg^{-1})	Skatole (mg kg^{-1})	Skatole/ indole	Indole (mg kg^{-1})	Skatole (mg kg^{-1})	Skatole/ indole	Indole (mg kg^{-1})	Skatole (mg kg^{-1})	Skatole/ indole	Indole (mg kg^{-1})	Skatole (mg kg^{-1})
Highest	0.302	1.71	15.4	0.16	0.186	3.2	0.602	0.901	4.0	0.057	0.177
Lowest	<0.015	<0.015	0.13	0	0	0.4	0.013	0.023	0.4	0.012	0.019
Average	0.035	0.25	3.7	0.084	0.101	1.4	0.151	0.201	1.8	0.029	0.046
n	137	137	137	15	15	15	20	20	20	14	14

methods described here are by no means rapid and they would not be suitable for sorting carcasses in an abattoir. Currently an isocratic, rapid, semi-automatic HPCL method is being developed for the determination of indole and skatole with a capacity of *ca.* 400 samples per day.

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Detection of heterocyclic aromatic amines in food flavours

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ABSTRACT

The successful determination of heterocyclic aromatic amines in trace amounts (ng/g) in processed flavours depends largely on the detection sensitivity and selectivity. As part of a systematic study of possibilities for the determination of these mutagenic amines, different detection modes in high-performance liquid chromatography were evaluated. Depending on the quality of the clean-up the identification of a peak by UV spectrophotometry (diode-array detection) might be necessary for a higher level of confidence. UV detection with gradient elution is the only method for the separation and determination of all the amines formed. However, electrochemical detection gives the best signal-to-noise ratio for isocratically eluted mutagenic amines.

INTRODUCTION

Heating of protein-rich food may lead to the formation of trace amounts of heterocyclic aromatic amines [1,2] (Fig. 1). These compounds are known to be mutagenic and potentially carcinogenic and they may be present in trace amounts in Maillard- and Amadori-type processed flavours. These browning-type food flavours are used in meat, cacao, bread and many other culinary flavours. They are very complex mixtures, with a high content of fat, and are commercially available as liquids, pastes or spray-dried powders.

As part of a systematic study of the determination of these heterocyclic amines in processed flavours, we evaluated the different possibilities for their detection in high-performance liquid chromatography (HPLC). Various methods for the determination and identification of these heterocyclic aromatic amines have been described. Liquid chromatography–mass spectrometry (LC–MS) [3–5], gas chromatography–mass spectrometry (GC–MS) [6] and HPLC with UV absorbance [7], electrochemical [8] and fluorescence detection have been successfully used, some for model systems only and others for cooked food products. Our objective was to find a simple but reliable method using HPLC as the separation technique and a suitable detection device for quantitative measurements of these mutagenic amines in processed food flavours.

LC–MS would be the preferred detection method for the highest selectivity and best on-line identification of mutagenic amines, but it is an expensive technique and may not be available in most laboratories. GC or GC–MS [9] is only possible with volatile derivatives of the mutagenic amines, otherwise these molecules tend to elute as broad, tailing peaks and therefore can not be detected in low concentrations. However, good derivatization reactions are possible only with some of the heterocyclic aromatic amines and a general method does not exist [10]. Incomplete derivatization leads to low sensitivity and non-reproducible results. Consequently the GC methodology was not developed further. In this paper we report the results obtained by using various HPLC detection methods; results of UV absorbance, electrochemical and fluorescence detection of these amines in food flavours are discussed.

EXPERIMENTAL

Materials

Aminoimidazoquinolines (IQ and MeIQ), aminoimidazoquinoxalines (MeIQx and DiMeIQx), aminopyridoindoles (Trp-P-1 and Trp-P-2) and aminopyridoimidazoles (Glu-p-1 and Glu-P-2)

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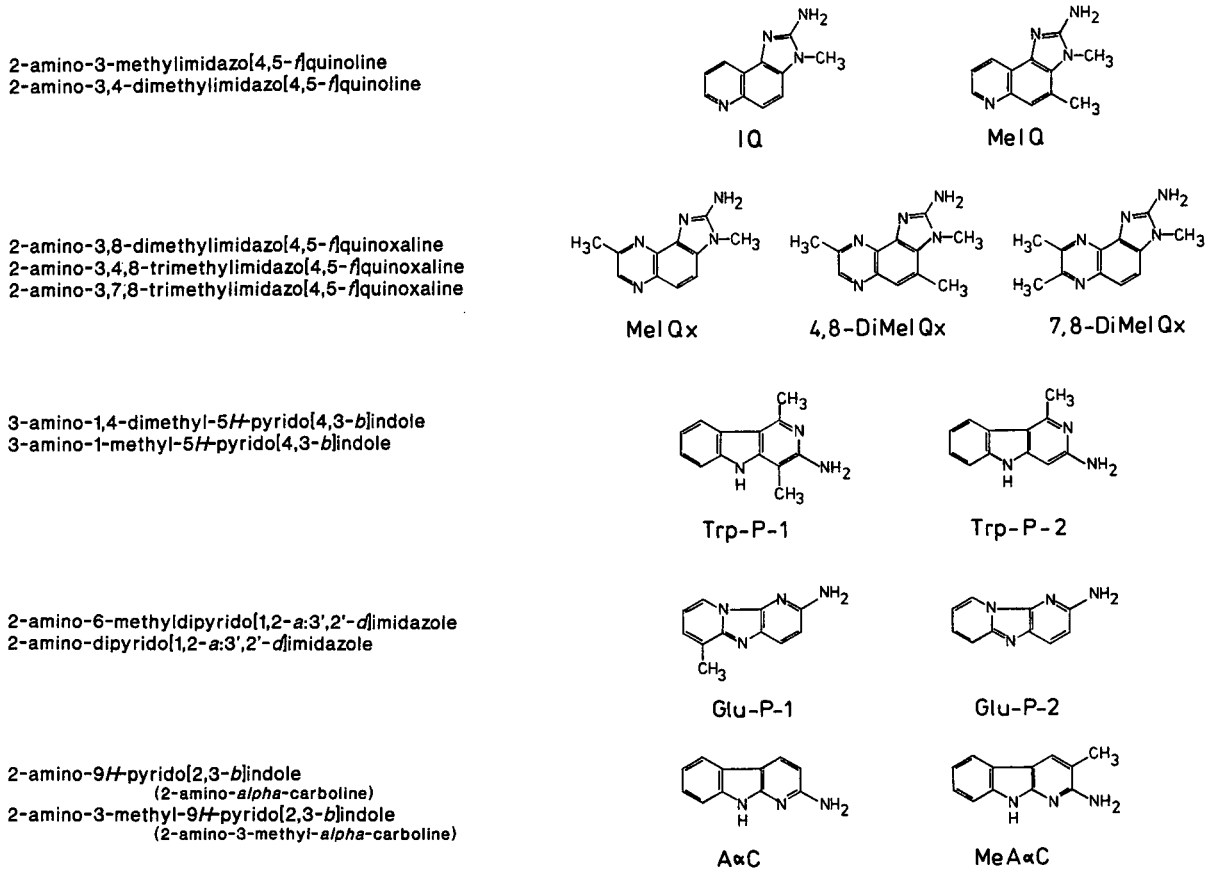

Fig. 1. Structures of heterocyclic aromatic amines.

were obtained from Toronto Chemical Research (Toronto, Canada). Their structures are shown in Fig. 1.

The reagents were of analytical-reagent grade (Fluka, Buchs, Switzerland) and the solvents (Rathburn Chemicals, Walkerburn, UK) were used without further purification. Water was purified in a UHP-2 system from Elgastat (Kleiner, Wohlen, Switzerland).

High-performance liquid chromatography

The column used for isocratic elution was a LiChrosorb RP-Select B, 5 μm (250 × 4.0 mm I.D.) from Merck (Darmstadt, Germany) operated at room temperature. Injections were made manually using a 10-μl loop injector (Valco, Schenkon, Switzerland). The mobile phase was a mixture of 0.1 *M* ammonium acetate solution (pH 4.5) (70–80%) and methanol–acetonitrile (1:2) (30–20%) pumped at 1.0 ml/min. Conditions for gradient elution are given in the legend to Fig. 4.

Detectors

The electrochemical detector was an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA), equipped with a glassy carbon electrode and operated in the oxidative mode. The applied potential was 950 mV and the typical offset was about 20 nA.

A Spectroflow 757 single-wavelength UV absorbance detector (Kratos, Ramsey, NJ, USA) with a cell volume of 12 μl was operated at a wavelength of 263 nm, with the highest sensitivity range of 0.005 a.u.f.s.

A Model 650-10LC fluorescence detector equipped with a flow cell of 15-μl volume was ob-

tained from Perkin-Elmer (Norwalk, CT, USA). The excitation wavelength was set at 360 nm and emission was measured at 450 nm.

An HP 1040A UV diode-array detector (Hewlett-Packard, Waldbronn, Germany) with an 8-μl cell was used. Two wavelengths were used to monitor the effluent simultaneously at 263 and 360 nm. Spectra of peaks were recorded from 210 to 400 nm.

Standard solutions

The standard solutions used for the determination of the absolute sensitivity and for showing the detection selectivity were prepared in the mobile phase. The composition of the standard mixture was selected to show the best possible use of the detector.

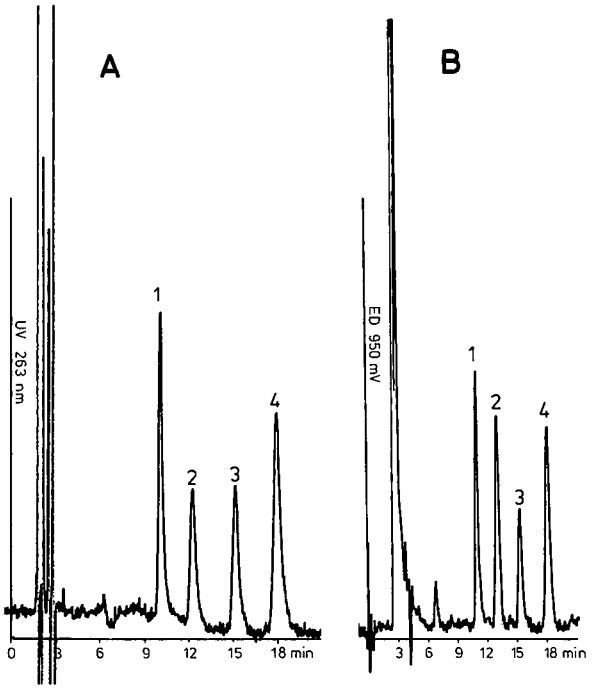

Fig. 2. HPLC of a standard solution of (1) MeIQx, (2) IQ, (3) 7,8-DiMeIQx and (4) 4,8-DiMeIQx. Column: LiChrosorb RP-Select B, 5 μm (250 × 4 mm I.D.). Mobile phase: mixture of 80% 0.1 *M* ammonium acetate solution (pH 4.5) and 20% methanol–acetonitrile (1:2). (A) UV detection at 263 nm: 1.35 ng of MeIQx, 1.13 ng of IQ, 1.04 ng of 7,8-DiMeIQx and 1.82 ng of 4,8-DiMeIQx. (B) Electrochemical detection at 950 mV: 270 pg of MeIQx, 226 pg of IQ, 208 pg of 7,8-DiMeIQx and 264 pg of 4,8-DiMeIQx.

Sample and sample preparation

A commercially available meat extract, as used in households and food industries, was chosen as a reference, to be able to compare the efficiency of the clean-up with results in the literature. The sample was a Maillard-type beef flavour. Maillard processed flavours contain large amounts of fat, which makes the sample preparation very difficult. Therefore, an efficient clean-up procedure is essential.

For the meat extract, the clean-up procedure described by Gross and Gruter [11,12] was used, with two solid-phase extraction steps. However, this clean-up is not sufficient for processed food flavours and additional steps had to be developed. To remove the large amount of fat we used Soxhlet extraction of the spray-dried product, placed the extract on Kieselgur (Extrelut; Merck) and extracted with diethyl ether prior to elution of the amines. Affinity chromatography on a copper phthalocyanine complex [13] (C.I. Reactive Blue 21; Hoechst, Frankfurt a.M., Germany), was an additional separation step to exclude other matrix components,

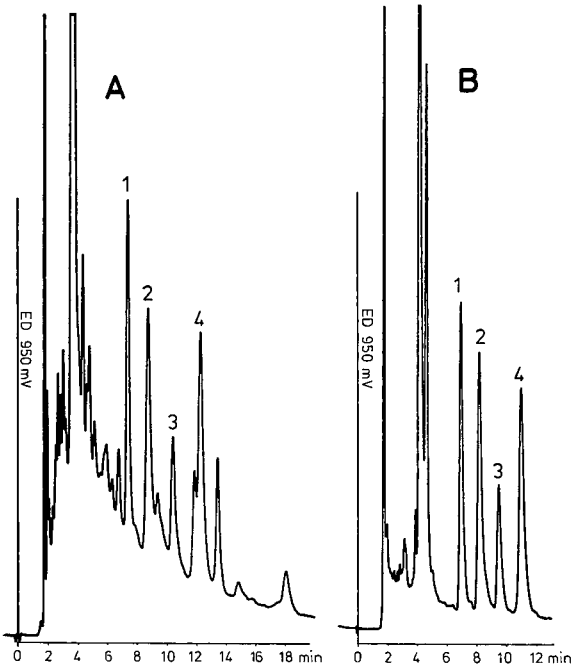

Fig. 3. Analysis of processed meat flavours spiked with 40 ng/g of (1) MeIQx, (2) IQ, (3) 7,8-DiMeIQx and (4) 4,8-DiMeIQx. Column and mobile phase as in Fig. 2. Electrochemical detection at 950 mV. (A) Beef flavour; (B) chicken flavour.

leading to a cleaner sample extract. Further work on this important step is in progress.

RESULTS AND DISCUSSION

UV absorbance detection is by far the most popular method for monitoring the effluents from HPLC columns. As most of the heterocyclic aromatic amines have good absorbance characteristics between 260 and 270 nm, this was the first method to be evaluated (Fig. 2A). The sensitivity achieved with our instrumentation was about 200 pg, but the detection selectivity was too low for the determination of the amines in the matrix of processed flavours at the level of 5–10 ng/g. We therefore looked for alternatives.

Grivas and Nyhammar [14] compared the sensitivity of UV and electrochemical detection (ED) for IQ, MeIQ, MeIQx and 4,8-DiMeIQx and found a 2–3 times lower detection limit for ED. However, they applied ED to model reaction systems only. Takahashi *et al.* [8] used HPLC–ED for the determination of IQ and MeIQx in beef extracts with a detection limit of *ca.* 0.2 ng/g. A more recent paper

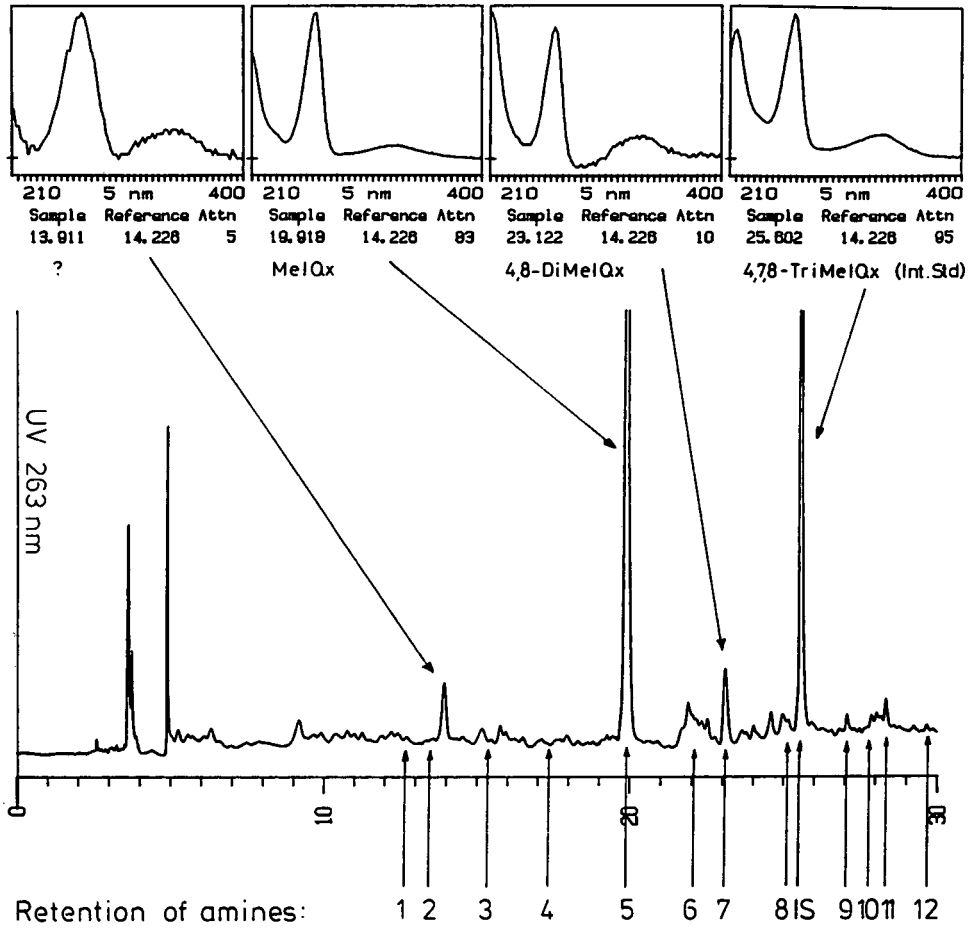

Fig. 4. Analysis of a natural meat extract. Column: TSK-gel, ODS-80 TM (250 × 4.6 mm I.D.). Mobile phase: A = 0.01 *M* triethylammonium phosphate (pH 3.2), B = 0.01 *M* triethylammonium phosphate (pH 3.6), C = acetonitrile, with tertiary gradient elution as follows: 0 min, 95% A, 0% B, 5% C; 15 min, 85% A, 0% B, 15% C; 16 min, 0% A, 85% B, 15% C; 20 min, 0% A, 75% B, 25% C; 30 min, 0% A, 45% B, 55% C. UV detection at 263 nm with spectra recording from 210 to 400 nm. The peak of MeIQx corresponds to an amount of 80 ng/g, 4,8-DiMeIQx was found in trace amounts (9 ng/g) and 4,7,8-TriMeIQx was used as internal standard. Peak identification: 1 = Glu-P-2; 2 = IQ; 3 = MeIQ; 4 = Glu-P-2; 5 = MeIQx; 6 = 4,8-DiMeIQx; 7 = norharman; 8 = harman; 9 = Trp-P-2; 10 = PhIP; 11 = Trp-P-1; 12 = A-α-C. Time scale in min.

by Billedeau *et al.* [15] confirmed the low sensitivity and reported not only on the optimization of the mobile phase for the separation of IQ, MeIQx, Glu-P-1, Trp-P-1 and Trp-P-2, but also on the effect of various potentials applied to the glassy carbon electrode on the detection sensitivity.

In our experiments, we found in addition to the expected low detection sensitivity of about 50 pg (Fig. 2B) a high detection selectivity for these amines in processed flavours (Fig. 3). For maximum detection sensitivity we applied a potential of 950 mV, using a glassy carbon electrode. This high potential reduces the selectivity to some extent, but gives the best results with our choice of mobile phase. However, it is impossible to use gradient elution in this high sensitivity range, which is a requirement for the determination of all eleven mutagenic amines in one run.

In addition to the limitations on the choice of the mobile phase, ED gives only the retention value for identification of a peak. UV detection with a diode-array instrument, capable of recording the UV absorption spectra of a peak, gives in addition to the retention the possibility of comparison of spectra for peak identification. Gross and Grüter [12] used this technique successfully in the analysis of meat extracts. Together with gradient elution and their suggested clean-up procedure, the determination of mutagenic amines in meat extract is feasible in the lower ng/g range (Fig. 4). For processed flavours, however, the detection selectivity is too low. Even with additional clean-up steps we were not able to meet a detection limit of less than 50 ng/g.

The imidazole-type amines show native fluorescence which can be used for highly sensitive and selective HPLC detection. The detection sensitivity is of the order of 1 pg and the limit of determination in processed beef flavour is below 0.5 ng/g. We therefore studied the possibilities of fluorogenic labeling of these heterocyclic aromatic amines. So far we have not been successful in derivatizing the common amino-group of the amines with a fluorescent reagent.

CONCLUSIONS

For a limited group of amines, ED is about five times more sensitive than UV detection. The main advantage of ED, however, is its selectivity. A good clean-up procedure is essential. The influence of the various steps in the sample preparation on the limit of determination of these heterocyclic aromatic amines in processed flavours is very high.

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Methods for the analysis of the saffron metabolites crocin, crocetins, picrocrocin and safranal for the determination of the quality of the spice using thin-layer chromatography, high-performance liquid chromatography and gas chromatography

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ABSTRACT

Saffron spice is the dry stigmata of *Crocus sativus* L. Methods have been developed for the determination of the quality of the spice using thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). TLC and HPLC gave comparable results for crocin and crocetins (colour principles), picrocrocin (bitter substance) and safranal (flavour). Similarly, the determination of safranal by GC was in agreement with analysis by TLC and HPLC. Separation of the constituents was achieved by silica gel G TLC using an *n*-butanol–acetic acid–water (4:1:1) system. The resolution of crocin, crocetins and picrocrocin by HPLC was obtained using a Shimadzu 15-cm CLC-ODS column with 20–80% acetonitrile in water as the eluent; for safranal an isocratic run with 76% acetonitrile in water was suitable. GC was adopted only for the determination of safranal using a Shimadzu 5% SE-30 column. HPLC was most suitable for the detection of adulterants and was simpler and more efficient for quality analysis. The TLC method was time-consuming and also gave an overestimation of the colour principles.

INTRODUCTION

Commercially used saffron is the dried stigmata of *Crocus sativus* L. It is popular because of its delicate aroma and attractive colour and is used as a food additive [1]. Saffron is cultivated in countries such as Spain, Italy, Iran, Switzerland and India. In India, its cultivation is restricted to the Himalayan state of Jammu and Kashmir. *Crocus sativus* plants require strict agroclimatic conditions for their growth, which influence the quality of the spice. The harvesting of the stigmata is a labour-intensive process and requires processing of 150 000 flowers for 1 kg of spice. This spice costs around $2000/kg in the international market.

Samples obtained from different geographical locations and from different processing methods are expected to show variations in quality with respect to colour (crocin and crocetins: carotenoid derivatives), flavour (safranal: a monoterpene) and the bitter principle (picrocrocin: a monoterpene glucoside). Moreover, due to the high cost of saffron, adulteration is rampant in India and the international market. The determination of the quality of saffron is an important consideration for the spice industry and for consumers. Of the various analytical methods described, the ISO recommended a thin-layer chromatographic (TLC) method for qualitative analysis of saffron [2]. However, this method is inadequate for grading saffron with respect to its vital constituents. The qualitative methods adopted for the determination of the colour and flavour profiles have been reviewed by Sampathu *et al.* [3]. The colour component is determined using a 1.5% potassium dichromate solution as a reference [4]. A determination method for flavour has been developed using gas chromatography (GC) [5] and spectrophotometry [6]. The determination of crocin,

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picrocrocin and safranal has been carried out by high-performance liquid chromatography (HPLC) [7,8]. No other methods have been published for the determination of these constituents.

The use of analytical methods for grading saffron has not been developed. In this work attempts have been made to develop sensitive methods for grading and assessing purity.

EXPERIMENTAL

Standards

An authentic sample of safranal was obtained from Malti-chem Research Centre (Baroda, India). An extract from the fruits of *Gardenia jasminoides* [9], collected from Wayanad District, Kerala, India, was used as the standard for crocin. Authentic crocetin was obtained from Sigma (St. Louis, MO, USA) (Cat. No. C 3398). The authenticity of the crocin standard was confirmed by the formation of a violet colour on the addition of concentrated H_2SO_4 and a green colour on the addition of concentrated HNO_3 [3]. All the reagents used were from SD Fine Chemicals (Boisar, India) unless stated otherwise.

A picrocrocin spot of R_F 0.32 was obtained by TLC [2] of the authentic saffron extract [in 80% (v/v) ethanol] using *n*-butanol–acetic acid–water (4:1:1). Picrocrocin was identified under UV light. The picrocrocin spot was eluted in 80% ethanol and concentrated to a known volume.

Source of saffron and extraction procedures

Saffron stigmata were obtained from fields at Pampore valley, Kashmir, India. Saffron samples were obtained from the local market. A 100-mg mass of dry stigmata was extracted with 5 ml of cold 80% (v/v) ethanol in a pestle and mortar, centrifuged at 5000 *g* for 10 min and washed twice with 5 ml each of the same solvent. The supernatant was used for further analysis by spectrophotometric and TLC procedures.

Thin-layer chromatography

Plates impregnated with approximately 0.25-mm-thick silica gel G were used for all the analyses. The plates were activated before use by heating at 100°C for 1 h. An aliquot of the alcoholic supernatant (0.1 ml) was spotted onto the plates. The developing chamber was saturated with the solvent *n*-butanol–acetic acid–water (4:1:1) for 6 h before running at room temperature in the dark. Crocin and crocetins were visible in white light whereas picrocrocin was detected under a UV source (254 nm) as a dark brown fluorescing spot.

For the determination of safranal, 2 ml of the 80% (v/v) ethanol extract were diluted to 4 ml with distilled water and separated five times with 10 ml of diethyl ether. The ether layers were pooled, air-dried and the volume made up to 0.1 ml with diethyl ether. This extract was spotted onto TLC plates which were developed in a hexane–ethyl acetate (9:1) solvent system under the described conditions. The safranal spot was visualized as a dark reddish brown spot on spraying with 2,4-dinitrophenylhydrazine (DNPH) reagent. The reagent was prepared by dissolving 0.5 g of DNPH [Glaxo Lab. (I), Bombay, India] in 25 ml of methanol acidified with one drop of concentrated H_2SO_4 [10].

After the spots had been identified, they were eluted on a preparatory scale into known volumes of 80% (v/v) ethanol and analysed spectrophotometrically at their λ_{max} (specified below).

Spectrophotometry

The supernatant (1 ml) was diluted to 5 ml with 80% (v/v) ethanol for analysis using a Shimadzu (Tokyo, Japan) UV-160 A, UV–visible recording spectrophotometer. The absorption maxima and extinction coefficients for crocin and picrocrocin were 443 nm, 89 000 and 250.5 nm, 10 100, respectively [7]; λ_{max} for safranal was 308 nm through a 1-cm pathlength as reported previously [7]. A standard graph was prepared by measuring the absorption of safranal at 308 nm.

Sample preparation and HPLC conditions

The extract of authentic stigmata in 80% (v/v) ethanol was passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA) and eluted with 100% acetonitrile. The eluent was flash-evaporated to dryness using a RotaVapor (Buchi, Switzerland) and redissolved in 20% (v/v) acetonitrile in water before injection onto the HPLC column. This step was necessary to clean the extract and was more efficient than passing the samples through a 0.22-μm Millipore filter (Millipore, Bedford, MA, USA).

A Shimadzu HPLC LC-6A system integrated with a Shimadzu SCL-6A system controller and a

SPD-6AV UV–visible spectrophotometric detector was used for the analysis of all the compounds. A Shimadzu 15 cm × 4.9 mm I.D. CLC-ODS column with a $5 \cdot 10^{-8}$ cm pore size and $10 \cdot 10^{-8}$ cm particle size was used for the separation and identification of the compounds. A Rheodyne Shimadzu Model 7125 injector was used to inject 10 μl of the sample from a 20-μl Hamilton (Hamilton, Reno, NV, USA) straight-edge needle syringe onto the column. All data were recorded on a Shimadzu CR4A recording system.

Crocin, crocetins and picrocrocin were separated on a gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min at a flow-rate of 0.5 ml/min. Picrocrocin was detected at 250.5 nm and the colour principles at 443 nm.

The separation of safranal was attempted using the following two methods: (i) a gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min at a flow-rate of 0.5 ml/min, detection at 308 nm; (ii) an isocratic run with 76% (v/v) acetonitrile in water at a flow-rate of 0.5 ml/min, detection at 308 nm.

All solvents used were of HPLC grade and filtered through a 0.45-μm filter (Millipore). A standard graph of area *versus* concentration was prepared using the standards.

Gas chromatography of safranal

The ether extract of safranal, as prepared for the TLC procedure, was used for the analysis. A 10-μl capacity Hamilton beveled syringe was used to inject 1 μl of the sample.

A Shimadzu 5% SE-30, 3-m stainless-steel column was fitted on a Shimadzu GC-15A gas chromatograph with an online flame ionization detector. An isothermal run at 150°C with nitrogen as the carrier gas at a flow-rate of 30 ml/min was used. All data were recorded on a Shimadzu CR4A recorder.

Statistical analysis of the samples analysed by TLC, HPLC and GC were carried out using Duncan's multiple range test and Student's *t*-test [11,12].

RESULTS AND DISCUSSION

Thin-layer chromatography

Crocin, picrocrocin and crocetins could be resolved on TLC plates run in the solvent system *n*-butanol–acetic acid–water (4:1:1) (Table I). For quantitative purposes the four crocetins, identified at R_F values of 0, 0.41, 0.75 and 0.98 were considered as total crocetins. The identification of individual crocetins was not carried out. Safranal, however, remained at the origin when run in this solvent system (Table I). As it interfered with the resolution of one of the crocetin derivatives, another solvent system (hexane–ethyl acetate, 9:1) was tested. In this system, safranal was well resolved (Table I). Moreover, another extraction procedure had to be suitably adopted (as detailed under Experimental) to resolve safranal using hexane–ethyl acetate (9:1).

TLC spots corresponding to crocin, crocetins, picrocrocin and safranal were eluted in 80% (v/v) ethanol and determined spectrophotometrically at their λ_{max} (see under Experimental). An average value of five replicates is given in Table II.

Direct spectrophotometric determination of the crude extract [*i.e.* the stigmata extract in 80% (v/v) ethanol] at various λ_{max} values corresponding to crocin, crocetins, picrocrocin and safranal did not

TABLE I

TLC ANALYSIS OF SAFFRON METABOLITES

TLC plates impregnated with 0.25-mm-thick Silica gel C were run with various solvent systems.

Component	R_F		Mode of detection
	n-Butanol–acetic acid–water (4:1:1)	Hexane–ethyl acetate (9:1)	
Crocin	0.63	–	Visible light
Picrocrocin	0.32	–	UV light
Crocetins	0.98, 0.75, 0.41, 0	–	Visible light
Safranal	0	0.78	DNPH reagent

TABLE II

DETERMINATION OF IMPORTANT CONSTITUENTS OF SAFFRON STIGMATA USING DIFFERENT ANALYTICAL PROCEDURES

TLC: conditions as in Table I. Crocin, crocetins and picrocrocin were eluted in 80% (v/v) ethanol and safranal in diethyl ether. Eluents were determined spectrophotometrically at a λ_{max} of 443, 250.5 and 308 nm, respectively. HPLC: column CLC-ODS (150 mm × 4.9 mm I.D.) using a gradient run from 20 to 80% (v/v) acetonitrile in 20 min for crocin, crocetins and picrocin. For safranal an isocratic run at 76% (v/v) acetonitrile was used. The flow-rate in both instances was 0.5 ml/min. The retention times under these conditions were: crocin: 13.5 min; crocetins 14–18 min; picrocrocin, 8 min and safranal 6 min. GC: safranal was separated on a 5% SE-30 3-m stainless-steel column using nitrogen as the carrier gas at a 30 ml/min flow-rate and detected using a flame ionization detection system. Values are mean ± S.D.

Method	Crocin (mg%)	Crocetins (mg%)	Picrocrocin (mg%)	Safranal (mg%)		
TLC	5.54 ±0.16	2.20 ±0.16	10.18 ±0.4	0.80 ± 0.02 (*A*)		
HPLC	5.86 ±0.15	2.15 ±0.064	11.23 ±0.23	0.83 ± 0.008 (*B*)		
GC[a]	–	–	–	0.81 ± 0.013 (*C*)		
Student's *t*-test[b]	3.16*	0.643 (NS)	4.4**	3.39** (*A–B*)	1.2 (NS) (*A–C*)	2.55* (*B–C*)

[a] Analysis of crocin, crocetins and picrocrocin by GC was not carried out.
[b] NS = Not significant; * = significant at $p \leq 0.05$; ** = significant at $p \leq 0.01$.

yield reproducible results. Moreover, it resulted in an underestimation of the constituents and hence was not suitable for analysis. In contrast, TLC followed by spectrophotometry gave reproducible results.

High-performance liquid chromatography

A gradient run from 20 to 80% (v/v) acetonitrile was suitable for the resolution of crocin, crocetins and picrocrocin (Figs. 1 and 2). The retention times for these compounds were 13.5, 14–18 and 8 min, respectively. The solvents used in this study were similar to those reported by Himeno and Sano [7] who used a Hiber LiChrosorb RP-18 (particle size 5 μm, column size 250 mm × 4 mm I.D.) (Cica-Merck) column which resolved crocin and picrocrocin, whereas the resolution of safranal was not as distinct as the former two compounds. In the present study the experiment with a gradient run could not resolve safranal (Fig. 3). However, an isocratic run (Fig. 4) using 76% acetonitrile gave a distinct peak of safranal at 6 min retention time. The resolution of safranal obtained in the isocratic run using 76% acetonitrile was far superior and can be used for routine analyses.

Crocetins were determined by summing the peak areas of different crocetin peaks (Fig. 1). The

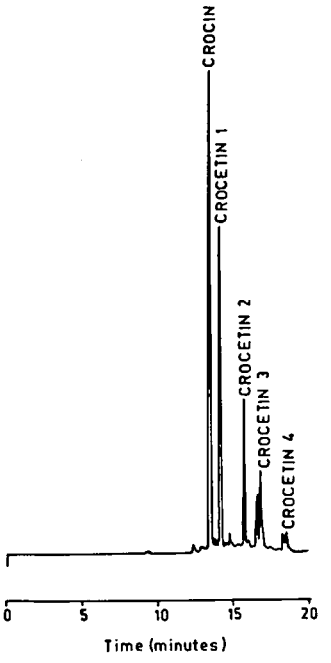

Fig. 1. HPLC profile of crocin and crocetins. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 × 4.9 mm I.D.); detection wavelength, 443 nm.

Fig. 2. HPLC profile of picrocrocin. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 250.5 nm.

concentrations of crocin, crocetin, picrocrocin and safranal obtained by the conditions standardized in this study are shown in Table II.

Gas chromatography

SE-30 and Carbowax columns were used with isothermal and thermal gradient programmes. It was found that an isothermal run at 150°C on an SE-30 column resolved safranal into a sharp single peak at a retention time of 3.6 min (Fig. 5). A quantitative determination of safranal was achieved using this method and the results were compared with those obtained by TLC and HPLC (Table II). GC determinations of the colour and bitter principles were not carried out as this required the derivatization of the compounds.

Statistical analysis of the three methods used showed that the results obtained by the HPLC method were significantly different from those of the TLC and GC methods (Table II), whereas the results

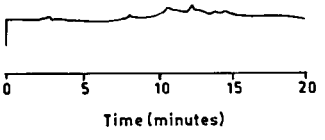

Fig. 3. HPLC profile of safranal (method i). Solvent, gradient run from 20 to 80% (v/v) acetonitrile, in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 × 4.9 mm I.D.); detection wavelength, 308 nm.

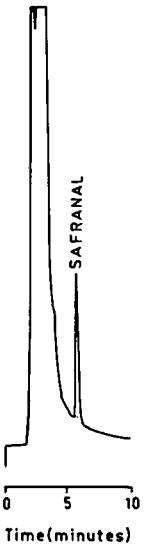

Fig. 4. HPLC profile of safranal (method ii). Solvent, 76% (v/v) acetonitrile in water; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 308 nm.

of TLC and GC were comparable. In general, the concentrations of all the metabolites determined by HPLC were higher than those determined by TLC and GC.

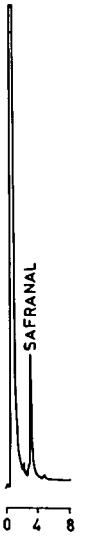

Fig. 5. GC profile of safranal. Temperature, 150°C; carrier gas, nitrogen (30 ml/min); column, Shimadzu SE-30 5% 3-m stainless steel; detector, flame ionization.

TABLE III

ANALYSIS OF MARKET SAMPLES OF SAFFRON

TLC and HPLC conditions as in Table II. Values are mean ± S.D.

Method	Crocin (mg%)	Crocetins (mg%)	Safranal (mg%)	Picrocrocin (mg%)
TLC	6.73 ± 0.12	0.81 ± 0.16	0.46 ± 0.01	0.48 ± 0.021
HPLC	4.8 ± 0.09	0.92 ± 0.14	0.4 ± 0.012	0.32 ± 0.02

Detection of adulteration

For the detection of adulteration in saffron, TLC and HPLC methods were tested (Table III). In many samples analysed by TLC there was overlapping of the adulterant spot and crocin, thereby rendering this method unsuitable for analysis, whereas the same sample analysed by the HPLC method gave a broad peak which was distinct from the crocin peak of the authentic sample (Fig. 6). Hence it was found that HPLC was most suitable method for the detection of adulterants in saffron.

CONCLUSIONS

TLC methods followed by spectrophotometry for the determination of crocin, crocetins, picrocrocin and safranal were reproducible. The GC method for the analysis of safranal gave comparable results to those obtained by TLC followed by spectrophotometry, whereas the HPLC method was sensitive and simpler as only one method of sample preparation and one set of solvents were required. The HPLC method was also the most suitable for the detection of adulterants.

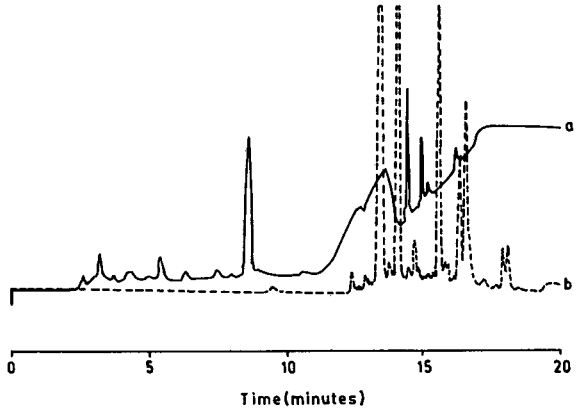

Fig. 6. HPLC analysis of saffron market sample. Solvent, gradient run from 20 to 80% (v/v) acetonitrile in water in 20 min; flow-rate, 0.5 ml/min; column, Shimadzu CLC-ODS (150 mm × 4.9 mm I.D.); detection wavelength, 443 nm. (a) Market sample; (b) stigmata from field.

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Separation and determination of 5-hydroxymethyl-2-furaldehyde and 2-furaldehyde in fruit juices by micellar electrokinetic capillary chromatography with direct sample injection

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ABSTRACT

The separation of 5-hydroxymethyl-2-furaldehyde (5-HMF) and 2-furaldehyde (2-FA), which are recognized indices of deteriorative changes in some commercially processed foods, was investigated by micellar electrokinetic capillary chromatography (MECC), employing sodium dodecyl sulphate as the anionic surfactant. The effects of micellar concentration, temperature and the addition of methanol or acetonitrile on the migration times and selectivity were investigated. MECC was successfully applied to the determination of 5-HMF and 2-FA in grapefruit juice by an internal standard method without any sample pretreatment.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC) is a rapidly developing, highly efficient separation technique based on micellar solubilization and electrokinetic migration in open-tubular capillaries, which is related to capillary electrophoresis [1–4]. A wide variety of applications of MECC in many areas of analytical chemistry have been reported, including the determination of drugs [5–7], antibiotics in plasma [8,9] and chiral compounds of pharmaceutical interest [10–12].

We are interested in the development of small-scale analytical methods for substances that are involved in the deterioration reaction occurring during food processing and storage. Two main chemicals that are generated in the non-enzymic browning process of fruit juices, 2-furaldehyde (2-FA) and 5-hydroxymethyl-2-furaldehyde (5-HMF), have been proposed as general indices of the deterioration of food quality during heating processes, *i.e.*, concentration, pasteurization or storage [13–16], although it was shown that they do not directly contribute to the perceptible off-flavour. Traditional approaches for the control and determination of 5-HMF and 2-FA include spectrophotometric and chromatographic procedures.

Spectrophotometric methods have been used for the determination of both 5-HMF and 2-FA in fruit juices [13,14], honey [15] and caramel [16]. These methods have the disadvantage of the instability of the coloured complex formed, the time required and the use of hazardous chemicals.

Chromatographic techniques used include thin-layer chromatography [17], gas chromatography [18] and, more recently, high-performance liquid chromatography (HPLC) [19–23] using UV detection at 280–285 nm. However, the presence of interfering peaks complicates the HPLC separation of 5-HMF and 2-FA and sample pretreatment, such as distillation [19], extraction [22] or clarification [20], is needed before HPLC.

This paper presents the results of a study of the electrophoretic conditions for the separation and determination of 5-HMF and 2-FA by MECC. The effects of micellar concentration, temperature and the addition to the running buffer of an organic modifier on migration times and selectivity were

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studied. The application of MECC to the identification and determination of 2-FA and 5-HMF in commercially processed grapefruit juice is also described.

EXPERIMENTAL

Equipment and procedure of MECC

Separations were carried out on a P/ACE 2100 HPCE instrument (Beckman Instruments, Fullerton, CA, USA) equipped with a fused-silica capillary cartridge (75 μm I.D. × 375 μm O.D.) with a total length of the capillary of 37 cm (30 cm to the detector). Prior to use the capillary was pretreated successively with 0.1 *M* HCl and 0.1 *M* NaOH for 30 min each, then rinsed with 0.2 *M* phosphate buffer (pH 7.5) and the running buffer. To maintain good peak shapes and reproducible migration times, the capillary tube was rinsed with 0.2 *M* phosphate buffer (pH 7.5) for 1 min and then with the running buffer for 2 min each time before a sample solution was injected. The capillary tube temperature was maintained at the experimental value to within ±0.1°C by means of a fluorocarbon liquid continuously circulated through the cartridge.

A deuterium light source with a 280-μm bandpass filter was used and absorbance was monitored at a range of either 0.006 or 0.010 a.u.f.s. Injection was made by nitrogen pressure for 2.5 s. All experiments were carried out applying a constant voltage of 10 kV with the anode at the inlet and the cathode at the outlet side. Data analysis and collection were accomplished using the Beckman Gold System software, version 6.01.

Materials

Sodium dodecyl sulphate (SDS) of electrophoresis purity reagent grade was purchased from Bio-Rad Labs. (Richmond, CA, USA); analytical-reagent grade phosphoric acid, hydrochloric acid and sodium hydroxide and HPLC-grade water, methanol and acetonitrile were all obtained from Carlo Erba (Milan, Italy).

5-Hydroxymethyl-2-furaldehyde, 2-furaldehyde and 2-furyl methyl ketone (2-acetylfuran) were purchased from Aldrich (Milwaukee, WI, USA). Micellar solutions were prepared by dissolving SDS in phosphate buffer, which was prepared by titrating a solution of 50 m*M* phosphoric acid with 0.1 *M* sodium hydroxide to pH 7.5. The solutions were filtered through a Type HA 0.22-μm membrane filter (Millipore, Bedford, MA, USA) and degassed by sonication before use.

All fruit juices, honey and spirits were purchased from a local store.

Procedure for quantitative analysis

A 2.0 mg/ml stock solution of 5-HMF and 2-FA in methanol–water (10:90, v/v) and 10 mg/ml of 2-furyl methyl ketone (2-FMK) in water as the internal standard solution were prepared daily. The stock solution was diluted to produce working standard solutions at five different concentrations within the range 0.1–50.0 μg/ml. An appropriate volume of internal standard solution was added to each solution to give a concentration of 10.0 μg/ml of 2-FMK. Calibration graphs were plotted based on the linear regression analysis of the peak-area ratios.

Grapefruit juice (exactly 25 ml) was diluted to 50.0 ml with water after the addition of the internal standard solution to give a concentration of 10 μg/ml of 2-FMK.

RESULTS AND DISCUSSION

Optimization of separation

We first investigated the effect of SDS concentration on the migration behaviour of the two furanic compounds. Both 5-HMF and 2-FA are non-ionic solutes and consequently in the capillary zone electrophoresis mode (*i.e.*, when the SDS concentration in the running buffer is zero) they migrated with almost the same velocity as methanol, which was the tracer of the electroosmotic flow.

On addition of SDS to the running buffer, the migration times of 5-HMF and 2-FA became longer than that of methanol, allowing the separation of the two samples. Above the critical micellar concentration (CMC), addition of SDS increases the micelle concentration with the monomer concentration remaining constant [24]. The plots in Fig. 1 show that the migration times of the solutes increased with increase in SDS concentration, although the electroosmotic flow was not changed significantly over the whole SDS concentration range. This means that the migration time of the two neutral solutes is proportional to the phase ratio of the micellar to the

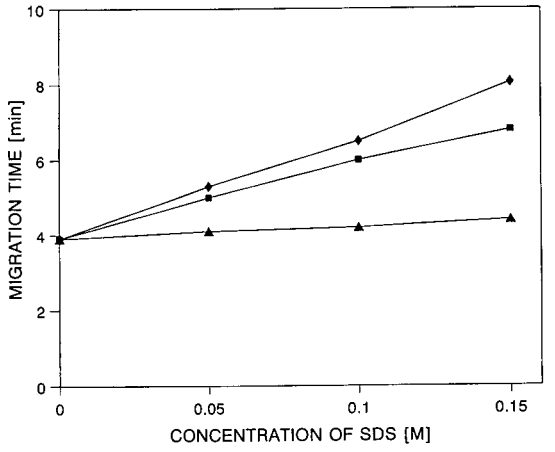

Fig. 1. Effect of SDS concentration on migration time. Buffer, 0.05 *M* phosphate (pH 7.5); capillary, fused silica, 370 mm × 0.075 mm I.D.; length of the capillary used for the separation, 300 mm; applied voltage, 10 kV; temperature, 25°C; detection wavelength, 280 nm. ■ = 5-HMF; ◆ = 2-FA; ▲ = methanol.

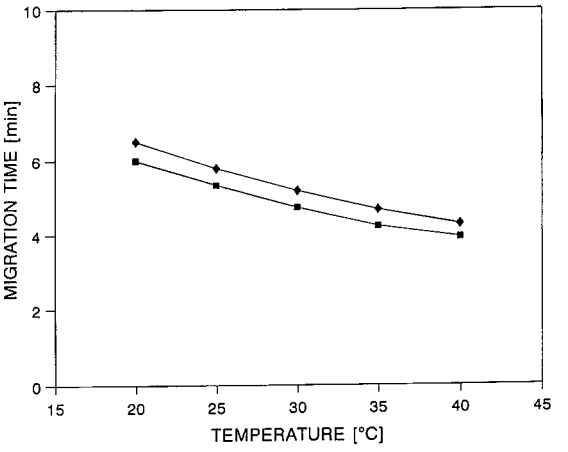

Fig. 2. Dependence of migration times on temperature. Running buffer, 0.050 *M* phosphate (pH 7.5) containing 0.10 *M* SDS. Other conditions and symbols as in Fig. 1.

aqueous phase, which is proportional to the micelle concentration.

The migration time of 2-FA was delayed more than that of 5-HMF with increasing SDS concentration, because 2-FA was more easily incorporated into SDS micelles owing to its higher hydrophobicity. At an SDS concentration of 0.10 *M* the two examined samples were well resolved within 7 min.

Increasing the capillary temperature from 20 to 40°C gave a large reduction in the migration times (see Fig. 2), whereas the resolution slightly decreased above 30°C. A decrease in the buffer viscosity, an increase in the CMC of SDS and changes in the distribution coefficient of the solutes between the micellar and aqueous phases were probably responsable for the observed results [2,25].

In addition to the parameters mentioned above, the effect of an organic modifier on the migration behaviour of 5-HMF and 2-FA was investigated by adding methanol or acetonitrile to the running buffer at concentrations up to 60%. These organic solvents were added to the running buffer on a volume/volume basis in a volumetric flask with appropriate amounts of SDS and phosphate buffer from stock solutions so that dilution to the mark gave the same concentration of SDS and phosphate ions in each solution.

As illustrated in Fig. 3, the migration times of 5-HMF and 2-FA increased with increase in either the methanol or acetonitrile concentration. However, the increase was much lower for the acetonitrile system. This effect is attributed to the increase in both the electroosmotic flow and the electrophoretic velocity of the micelles [26]. The electroosmotic flow is affected by variations in the viscosity and dielectric constants of the running buffer and by the change in the net charge of the capillary wall, which appear to be reduced with the addition of an organic modifier [27].

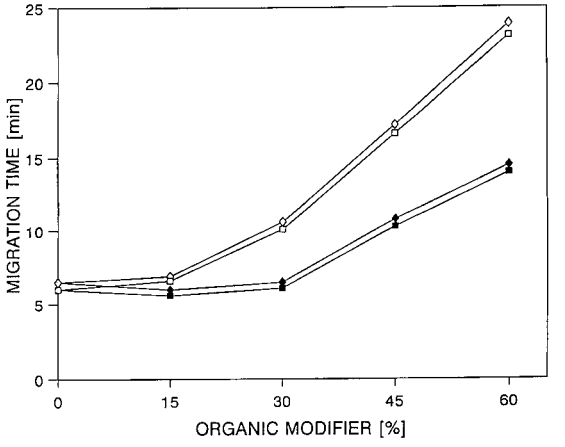

Fig. 3. Migration time as a function of the percentage of organic modifier. Open symbols, methanol; closed symbols, acetonitrile. □, ■ = 5-HMF; ◇, ◆ = 2-FA. Other conditions as in Fig. 2.

For 5-HMF and 2-FA, the migration order and selectivity were not influenced by the addition of methanol or acetonitrile, probably because these electrically neutral solutes have nearly the same structure. However, the peak broadening effect that occurred as the migration time of the two solutes increased with increase in the organic modifier concentration was larger in the acetonitrile than in the methanol system.

Qualitative and quantitative analysis

On the basis of the results reported above, a 0.050 *M* phosphate buffer solution of pH 7.5 containing 0.10 *M* SDS was selected for qualitative and quantitative analysis of 5-HMF and 2-FA in fruit juices. The temperature of the capillary cartridge was maintained at 20°C.

In order to examine the reproducibility of the migration times, the mean value, the standard deviation (S.D.) and the relative standard deviation (R.S.D.) of the migration time were calculated from the electropherograms obtained by five repeated injections of a sample solution containing equimolar amounts of 5-HMF and 2-FA and the internal standard. The results are reported in Table I and show that the R.S.D.s were better than 0.95% for the three compounds.

Quantification was performed by an internal standard method. A number of possible internal standards were explored for the simultaneous determination of 5-HMF and 2-FA. 2-FMK was selected as the internal standard as it was well resolved from both 5-HMF and 2-FA and met the following criteria which were evaluated in assessing a suitable

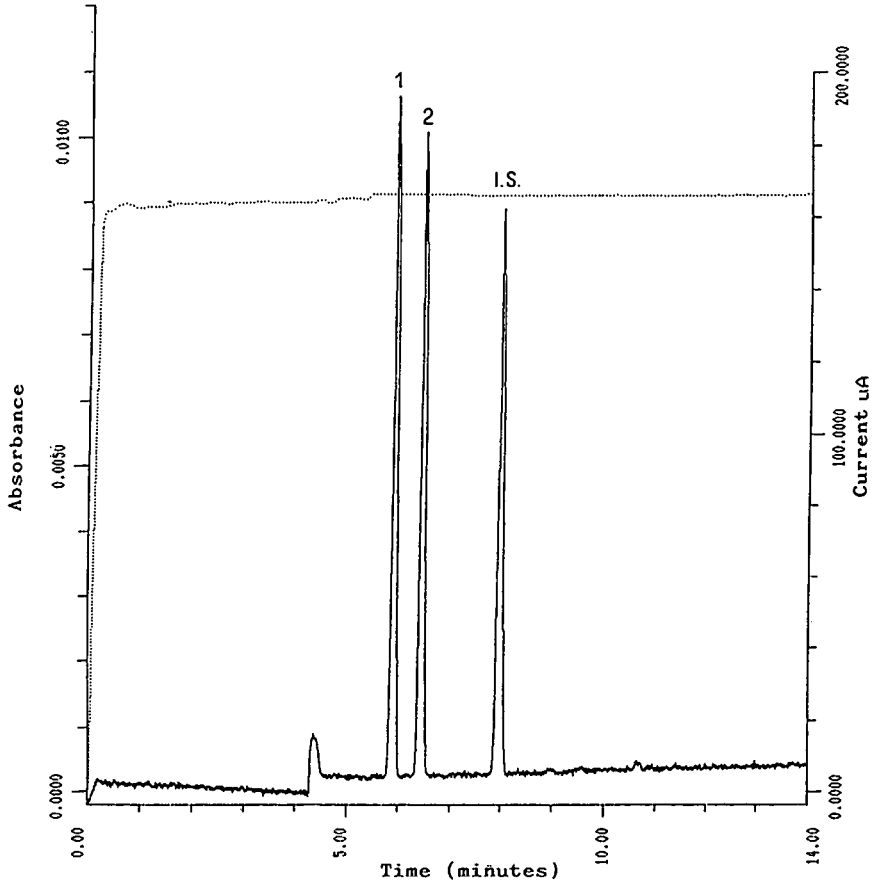

Fig. 4. Separation of (1) 5-HMF, (2) 2-FA and (I.S., internal standard) 2-FMK by MECC with 0.10 *M* SDS in 0.050 *M* phosphate buffer (pH 7.5). Capillary, fused silica (370 × 0.075 mm I.D.); length of capillary used for the separation, 300 mm; applied voltage, 10 kV; temperature, 20°C; detection wavelength, 280 nm.

TABLE I

REPRODUCIBILITY OF MIGRATION TIMES

Five repeated injections. Conditions as in Fig. 4.

Sample	Migration time (min)			R.S.D. (%)
	Individual values	Mean	S.D.	
5-HMF	5.95, 5.98, 6.04, 6.02, 5.93	5.98	0.046	0.77
2-FA	6.46, 6.50, 6.58, 6.55, 6.43	6.50	0.062	0.95
2-FMK	7.88, 7.91, 8.04, 8.02, 7.94	7.96	0.069	0.87

TABLE II

REPRODUCIBILITY OF PEAK-AREA AND PEAK-HEIGHT RATIO WITH RESPECT TO THE INTERNAL STANDARD

Five repeated injections. Conditions as in Fig. 4.

Sample	R.S.D. (%)	
	Peak-area ratio	Peak-height ratio
5-HMF	1.25	3.53
2-FA	0.58	2.40

candidate: absorption in the region of 280 nm, solubility in aqueous solution and chemical similarity to 5-HMF and 2-FA.

A typical electropherogram of these compounds together with the internal standard is shown in Fig. 4. The reproducibility of the determination of 5-HMF and 2-FA was investigated by repeated injection ($n = 5$) of an equimolar sample solution containing the internal standard. As shown in Table II, the peak-area ratio mode gave a higher reproducibility than the peak-height ratio mode. The calibration graphs for 5-HMF and 2-FA obtained by the

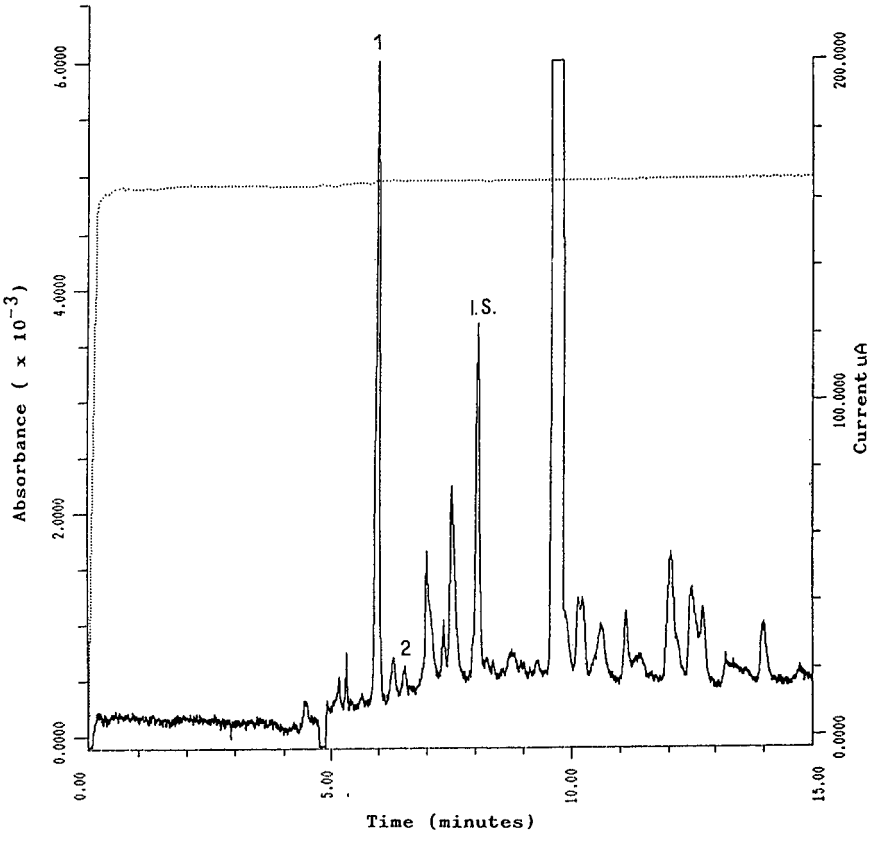

Fig. 5. Electropherogram of an industrial processed grapefruit juice. Conditions as in Fig. 4. (1) 5-HMF; (2) 2-FA; (I.S.) 2-FMK.

TABLE III

REPRODUCIBILITY OF THE DETERMINATION OF 5-HMF AND 2-FA IN AN INDUSTRIAL PROCESSED GRAPEFRUIT JUICE

Conditions as in Fig. 4.

Analyte	Amount found (μg/ml)			R.S.D. (%)
	Individual values	Mean	S.D.	
5-HMF	15.406, 15.127, 14.960, 15.542, 14.881	15.123	0.212	1.400
2-FA	0.602, 0.628, 0.596, 0.622, 0.615	0.612	0.013	2.189

peak-area ratio method showed excellent linearity over the concentration range 0.1–50 μg/ml with correlation coefficients $r = 0.99982$ and 0.99985, respectively, and nearly passed through the origin.

Determination of 5-HMF and 2-FA in fruit juices

Both 5-HMF and 2-FA are recognized indicators of the quality deterioration of fruit juices during the heating process, *i.e.*, concentration, pasteurization or storage [28]. As an application, we determined the amount of 5-HMF and 2-FA in a commercial grapefruit juice. The grapefruit juice was directly injected onto the capillary tube without any sample pretreatment, except that it was filtered through a 0.22-μm membrane filter after addition of the internal standard solution. The assay results are summarized in Table III and a typical electropherogram is shown in Fig. 5.

CONCLUSIONS

This study has shown that MECC, employing SDS as the anionic surfactant, is an effective method for the qualitative and quantitative analysis of 5-HMF and 2-FA in grapefruit juice from the viewpoint of successful separation, high accuracy, high reproducibility and short analysis time. It is also notable that no sample pretreatment is necessary.

Preliminary attempts to apply this technique to other processed foods have indicated that it can be employed for the determination of 5-HMF and 2-FA in honey, spirits and other fruit juices and concentrates.

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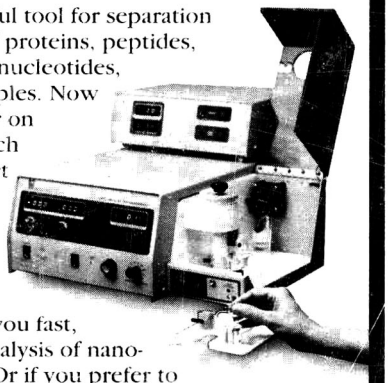

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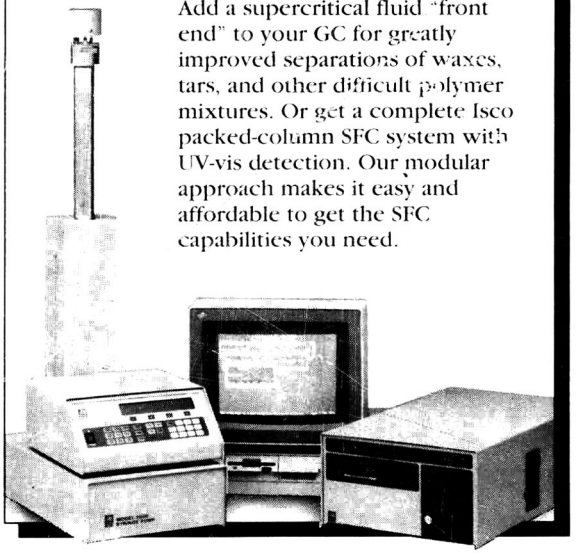

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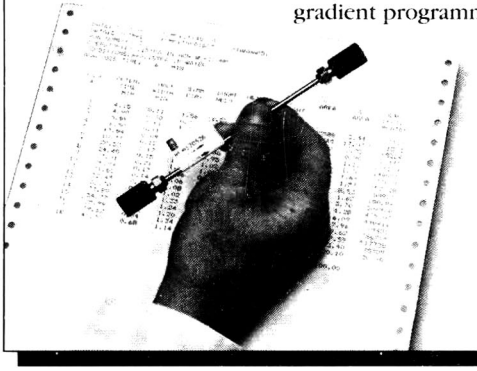

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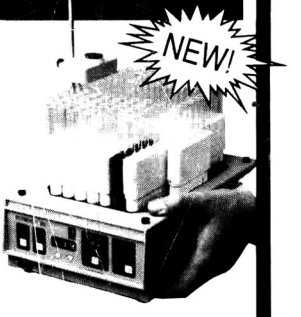

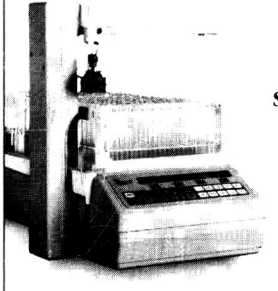

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