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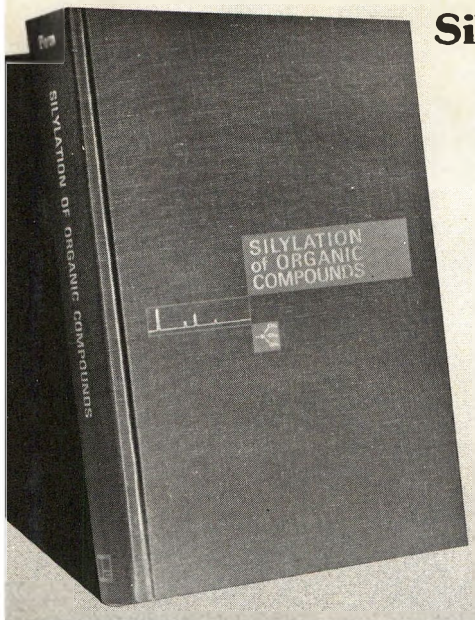

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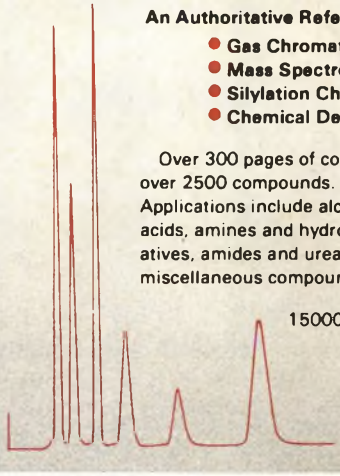

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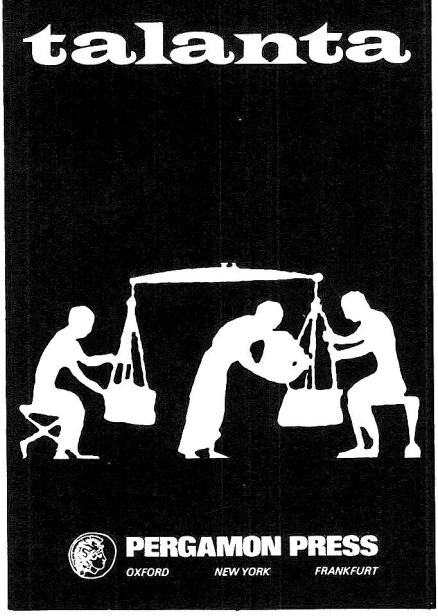

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REVIEW OF ANALYSIS OF GLUCOSINOLATES

Analytical Methodology for Determining Glucosinolate Composition and Content

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GRUFFYDD R. FENWICK
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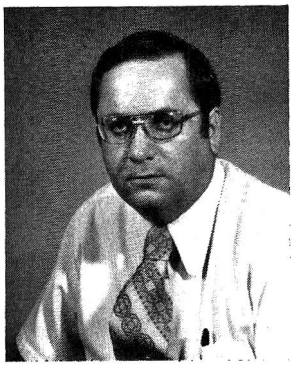
New analytical techniques and instrumentation and increased knowledge of the diversity and distribution of glucosinolates, the diversity of their enzymatically released products, and factors influencing their release, have led to significant advances in methodology for analysis of glucosinolates over the past three decades. However, many of the methods have been developed for specific agricultural crops or commodities and their particular glucosinolate compositions. They can only be applied to certain types of plant material or can detect and quantitate only certain glucosinolates or their products. Other methods have been designed to meet specific research, regulatory, or quality control requirements. This had necessitated sacrifice of either speed, simplicity, accuracy, precision, or the ability to distinguish different glucosinolates or their products. This review examines the methods available for sample preparation, identification, and quantitation of glucosinolates in light of current knowledge of their diversity, distribution, and chemistry. Consideration is given to the suitability of methods for rapid screening or precise, discriminating measurement, and to the standardization of methodology and reporting of results.

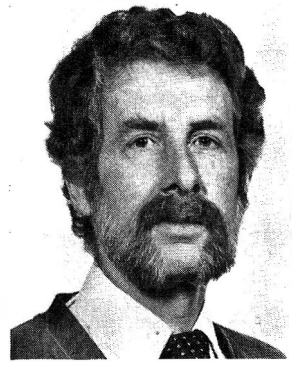
Glucosinolates are a uniform class of more than 90 compounds that occur in only 11 families of dicotyledonous plants (1). Most of the cultivated plants which contain glucosinolates belong to the Cruciferae family and especially to the *Brassica* genus. Glucosinolates are characterized by the fact that they co-exist, but are not in contact with, an enzyme called myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). When the structure of the plant material in which they reside breaks down, or is broken down in the presence of moisture, glucosinolates are hydrolyzed by myrosinase. In addition to glucose and sulfate, isothiocyanate, thiocyanate, oxazolidinethione, and nitrile hydrolysis products may be produced.

Glucosinolates are of interest because the enzymatically released aglucones are physiologically active compounds (2). They are responsible for the biting taste of important condiments (horseradish, mustard) and contribute to the characteristic flavors of many plants whose leaves (brussels sprouts, cabbage), flower buds (broccoli, cauliflower), stem (kohlrabi), or root (radish, rutabaga, turnip) are consumed as vegetables. When consumed by humans in small amounts as part of the normal diet, the enzymatically released products are esteemed. But when consumed by animals in larger amounts as part of their feed, the products may reduce palatability or be toxic. They restrict the use of several plants (kale, radish) for pasture, forage, or silage and the use of some major oilseed crops (mustard, rapeseed) as animal feed.

Until recently, determination of the glucosinolate content of agricultural crops or commodities was only possible by indirect measurement of one or another of the enzymatically released products. Over the past 3 decades, advances in instrumentation and increased knowledge of the diversity of the glucosinolates, their enzymatically released products, and fac-



D. I. McGregor is currently a research scientist specializing in the physiology and chemistry of rapeseed and mustard at the Agriculture Canada Research Station, Saskatoon, Saskatchewan. He received his graduate education from Purdue University, Lafayette, Indiana. He is a member of the Agricultural Institute of Canada, the Saskatchewan Institute of Agrologists, and the Canadian Institute of Food Science and Technology and is chairman of the Canadian Associate Committee of the International Standards Organization Oilseeds Subcommittee. Dr. McGregor has been interested in the analysis of glucosinolates for several years, particularly as it relates to the breeding of rapeseed for reduced glucosinolate content and to the identification of low glucosinolate (canola) rapeseed in commerce.



W. J. Mullin received his graduate education from the University of Alberta (Ph.D. in Food Chemistry) after working in the United Kingdom and Canada on the analysis of plastics and fuels. He is currently Team Leader of the Food Safety and Nutrition section of the Food Research Institute, Agriculture Canada. For several years, he was involved with the analysis of glucosinolates in vegetables and more recently with analytical methodology for vitamins in vegetables and cereals.

G. R. Fenwick received his B.Sc. (Honours) and Ph.D. in Chemistry from the University of Wales, University College, Swansea. After holding Synvar and Medical Research Council Fellowships in Palo Alto, CA, and Sheffield, UK, respectively, he joined the Agricultural Research Council's Food Research Institute at Norwich in 1971, and is currently Principal Scientific Officer in charge of the Natural Toxicants Group, as well as Honorary Lecturer in Chemistry at the University of East Anglia. Dr. Fenwick is a Fellow of both the Institute of Food Science and Technology and the Royal Society of Chemistry. In the latter Society, he is Honorary Secretary of the Food Chemistry Group and a member of the Federation of European Chemical Societies Working Party on Food Chemistry. While retaining an interest in mass spectrometry, the subject of his doctoral thesis, Dr. Fenwick's current research activities are centered in the area of food safety and natural toxicants.

tors influencing their release have led to the advent of a multiplicity of analytical methods. Most of the products of myrosinase hydrolysis, including isothiocyanates, thiocyanate ion, oxazolidinethiones, nitriles, sulfate, and glucose, have been used as the basis of measurement. But of particular interest have been methods involving measurement of the hydrolysis products, because it is these and not the glucosinolates per se which are responsible for the flavors as well as most of the biological effects. In recent years, methods have also been developed for determining glucosinolates, or derivatives thereof, which do not involve myrosinase hydrolysis. These methods are popular because they avoid problems associated with myrosinase hydrolysis. Some are popular because they can also separate and individually quantitate many specific glucosinolates.

For an analyst new to the field, the choice of a suitable method for determining glucosinolate composition or content may be bewildering. Ideally, an analytical method should be rapid, simple, sensitive, precise, and accurate and discriminate between all compounds of interest. Rarely are all of these attributes found in one method. To meet specific research, regulatory, or quality control requirements, it is often expedient to sacrifice some in preference for others. The nature of the plant material to be analyzed and the diversity and distribution of the glucosinolates may also influence the choice of method. As analytical methodology has evolved and improved, some methods have become obsolete. But others are still useful for determining either the composition or content of glucosinolates in certain crops and products because of their simplicity or low cost.

The contribution of glucosinolate breakdown products to the flavor and biological effects of agricultural crops and commodities has recently been reviewed (2). In the present review, analytical methodology for determining glucosinolate composition and content of these crops and commodities is critically examined in light of current knowledge of the diversity, distribution, and chemistry of these compounds. Sample preparation, methods for glucosinolate identification, and both direct and indirect methods for determining glucosinolate content are examined. Consideration is given to the suitability of direct methods for rapid screening or precise, discriminating measurement. Standardization of methodology for regulatory and quality control, and of reporting results, is also considered.

Background

Structure

The structure for virtually all glucosinolates (Figure 1, I) was first proposed, and later verified with the synthesis of benzyl glucosinolate (glucotropaeolin), by Ettlinger and Lundeen (3, 4). The sugar moiety, with a single exception, has been found to be glucose. This exception, reported in radish (*Raphanus sativus* L.) seed, contains a 6-sinapolythioglucose moiety (5). Glucosinolates are anions frequently isolated from plants as potassium salts, although salts of sinapine (the choline ester of sinapic acid) occur widely among the Cruciferae (6).

Nomenclature

The first 2 glucosinolates discovered were given the now classical names sinalbin and sinigrin. Some glucosinolates discovered subsequently have received trivial names which likewise do not describe their structure. These names were derived from the Latin name of the plant from which the glucosinolate was first isolated, prefixed with "gluco," and suffixed with "in." Thus, glucocheirolin from *Chieranthus* and gluconasturtiin from *Nasturtium officinalis* (water cress). As the number of isolated glucosinolates increased, this trivial approach to naming became unwieldy. The complexity of glucosinolate structures made a fully systematic scheme impracticable. To compromise, Ettlinger and Dateo (7) introduced a semi-systematic system of naming in which all members of the class (Figure 1, I, R = H) are called glucosinolates and to which is added a prefix that chemically describes the variable part of the molecule (R). Thus, glucocheirolin becomes 3-methylsulfonylpropyl glucosinolate, and gluconasturtiin becomes 2-phenylethyl glucosinolate.

In the past, glucosinolates have been collectively called thioglucosides, mustard oil glucosides, and isothiocyanate-producing glucosides because of their structure and their ability to produce isothiocyanate. In early literature, isothiocyanates were referred to as mustard oils because of their origin. More recently, the term glucosinolate has been preferred in keeping with the semi-systematic approach to naming.

Diversity

4-Hydroxybenzyl glucosinolate (sinalbin) and allyl (2-propenyl) glucosinolate (sinigrin) were first isolated about a century and a half ago from yellow mustard (*B. hirta* Moench)—also known as white mustard (*Sinapis alba* L.) (8)—and black

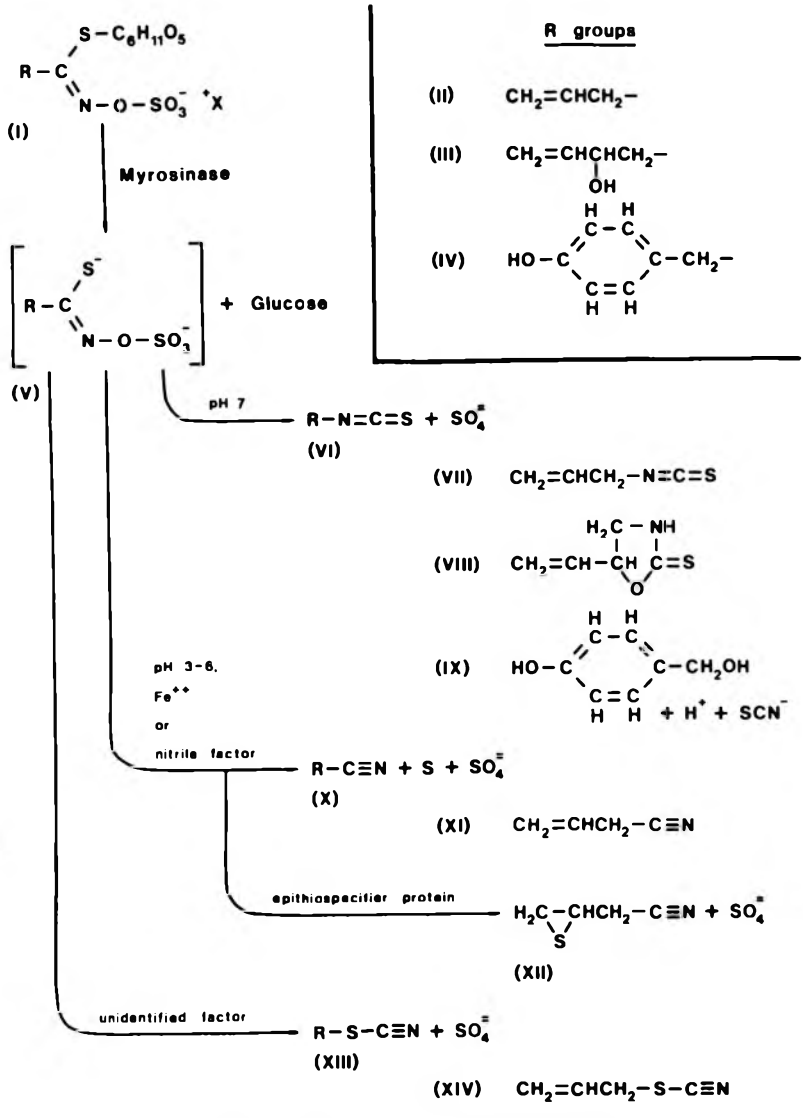


Figure 1. Products of myrosinase hydrolysis.

mustard (*Brassica nigra* (L.) Koch) (9), respectively. Over the next century, only one additional glucosinolate, 3-methylsulfonylpropyl glucosinolate (glucocheirolin), was isolated and identified (10). However, by 1948, 9 glucosinolates were known and, by 1960, the number had increased to 30 (11). The number now exceeds 90 (2).

Detailed description of individual glucosinolates with reference to their discovery has been reviewed elsewhere (12, 13, 14). A wide range of glucosinolates occurs naturally including those possessing simple and branched alkyl and alkenyl, hydroxyalkenyl, methylthioalkyl, methylsulfinylalkyl, methylsulfonylalkyl, monoketoalkyl, aromatic, and heterocyclic side chains.

Distribution

Glucosinolates have been found in practically every species of the plant families Cruciferae, Capparaceae, Tovariaceae, Resedaceae, and Moringaceae. They have also been found in Limnanthaceae, Caricaceae, Tropaeolaceae, Gyrostemonaceae, Salvadoraceae, and Euphorbiaceae. Current knowledge of their distribution within these families has recently been reviewed by Kjaer (1).

The majority of cultivated plants which con-

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tain glucosinolates belong to the Cruciferae family and specifically to the genus *Brassica* within this family (13, 15). Mustard seed used as a condiment is derived from *B. nigra*, *B. juncea* (L.) Coss., and *B. hirta* species. Vegetable crops include cabbage, cauliflower, broccoli, brussels sprouts, turnip, and rutabaga of the *B. oleracea* L., *B. campestris* L., and *B. napus* L. species. Rapeseed oil derived from the *B. campestris* and *B. napus* species is one of the world's major edible oils. The meal remaining after oil extraction is used as an animal feed. Kales of the *B. oleracea* species are used for forage, pasture, and silage.

In the genus *Brassica* (16, 17), and other genera (2, 18), considerable differences in the abundance of individual glucosinolates can be readily detected between species, individual plants within a species, and between individual parts of the same plant. Even within the same plant part, glucosinolate levels can vary with development, usually being highest during the period of most active growth (19–21). Such differences are thought to be only quantitative, not qualitative (19–22). Thus, McGregor (23) has found, contrary to earlier reports, that indole glucosinolates are not restricted to the vegetative parts of *Brassica* plants, but are also present in the seed.

Despite the large number of glucosinolates which have been identified, most species are thought to contain only a few, and even fewer in abundance. However, the use of methods which can detect only specific types of glucosinolate breakdown products, most notably volatile isothiocyanates, has probably resulted in a somewhat restricted view of glucosinolate distribution. In a recent study of the volatiles of horseradish, using combined capillary gas chromatography–mass spectrometry (24), 30 glucosinolates were detected, although the evidence for some of the minor components must be regarded as tentative. With the application of newer methods, and in particular methods which can detect intact glucosinolates or derivatives thereof, knowledge of glucosinolate distribution can be expected to grow.

Myrosinase Hydrolysis

In the presence of moisture, glucosinolates are hydrolyzed by the enzyme myrosinase to yield an unstable aglucone (Figure 1, V) which then breaks down to yield a range of products. Only one enzyme is involved, although isoenzymic forms have been reported (25). The nature of the glucosinolate, the reaction conditions, and the cofactors present in the plant tissue, some of which are as yet unidentified, determine which of the various products will be formed.

When myrosinase preparation is added to an aqueous solution of a glucosinolate (I), glucose is released and a reactive aglucone (V) is formed which at neutral pH undergoes a Lossen-type rearrangement to produce an isothiocyanate (VI). Most glucosinolates give rise to a stable isothiocyanate. Allyl glucosinolate (II), for example, yields allyl isothiocyanate (VII). However, glucosinolates which possess a beta-hydroxyl group in their side chain, such as 2-hydroxy-3-butenyl glucosinolate (progoitrin/epiprogoitrin) (III), give rise to an isothiocyanate that spontaneously cyclizes to form an oxazolidinethione, such as 5-vinyl oxazolidine-2-thione (VIII) (13, 18). Some aromatic and heterocyclic glucosinolates, notably 4-hydroxybenzyl glucosinolate (26) and, presumably, the indole glucosinolates (27, 28) give rise to isothiocyanates which are unstable at neutral or alkaline pH and break down to release inorganic thiocyanate ion. With 4-hydroxybenzyl glucosinolate (IV), 4-hydroxybenzyl alcohol is formed in addition to inorganic thiocyanate ion (IX).

When myrosinase preparation is added to a glucosinolate solution under weakly acidic conditions (29, 30) or in the presence of ferrous ion (31, 32), a nitrile (X), such as allyl nitrile (XI), and el-

emental sulfur may also be formed. When fresh plant tissue is autolyzed, that is, the glucosinolates are hydrolyzed by the endogenous myrosinase and cofactors, nitriles may predominate. In the case of 2-hydroxy-3-butenyl glucosinolate in rapeseed or crambe (*Crambe abyssinica* Hochst. ex R. E. Fries), it has been shown that myrosinase per se is not responsible for the difference in products. Rather the presence of an as yet unidentified cofactor favors nitrile production (33). Tookey and Wolff (31) found aged crambe meals to be less likely to produce a nitrile but that this could be overcome by the presence of mercaptoethanol during hydrolysis, indicating that the cofactor was sensitive to oxidation. Like myrosinase, the factor is also relatively labile to heat and to polar organic solvents. Heat treatment of rapeseed and crambe meals has been shown to favor isothiocyanate production at the expense of nitriles (33). Indeed, increasing the hydrolysis temperature alone was shown to favor isothiocyanate formation (33). Treatment of rapeseed meals with ethanol has been shown to increase the nutritional value (34), presumably by inactivating the nitrile factor and thus reducing the formation of nitriles by autolysis.

One cofactor, which can influence the nature of the hydrolysis products formed, has been isolated from various sources and has been shown to be a protein (18, 35–37). Termed an epithiospecifier protein, it is, by itself, devoid of myrosinase activity. However, when combined with myrosinase it directs the hydrolysis of alkenyl glucosinolates, such as allyl glucosinolate (II), to yield an epithionitrile such as 1-cyano-2,3-epithiopropane (XII). Studies with 2-hydroxy-3-butenyl glucosinolate and myrosinase isolates have shown that both ferrous ion and epithiospecifier protein are required for epithionitrile formation, indicating that the action of the epithiospecifier protein is superimposed on the ferrous ion-induced nitrile formation (18). When glucosinolates are autolyzed, presumably the epithiospecifier protein acts in combination with the previously mentioned nitrile cofactor.

Another as yet unidentified cofactor that can direct the hydrolysis of a glucosinolate to produce a thiocyanate (XIII) rather than an isothiocyanate may be present in some plants. For example, the allyl glucosinolate (II) in stinkweed (*Thlaspi arvense* L.) has been shown to produce predominantly allyl thiocyanate (XIV) when fresh tissue is crushed (38). It is yet to be ascertained whether the products are formed enzymatically or whether they are formed subsequently after the action of myrosinase (39). In this respect it is interesting that the cofactor leading to the formation of thiocyanates is labile to both heat and oxidation (18), a striking parallel to the formation of nitriles.

Sample Preparation

Removal of Substances Impeding Analysis

Analysis for glucosinolate composition or content is greatly facilitated if oil and moisture are first removed from seed and vegetation, respectively.

Because glucosinolates are hydrophilic compounds, seed oil is easily removed using nonpolar solvents. Petroleum ether (bp 30–60°C) is usually the solvent of choice, because this solvent is customarily used in commercial extraction of oilseeds. Although glycolipids and phospholipids are incompletely extracted, the remaining meal reflects the composition of the commercial product. Soxhlet or Twisselmann (Butt) type extraction can be used to extract small samples. However, the Swedish tube method (40), in which the sample is placed in a sealed stainless steel tube with solvent and steel balls and then shaken horizontally, can facilitate oil removal by combining the grinding and extraction into one operation. Glucosinolates or their hydrolysis products are easily extracted from the resulting finely divided meal. High-speed sonic grinders, such as the Polytron macerator, can also facilitate oil removal by combining the macerating and extraction into one operation. Throughput is higher due to the ease of cleaning the macerator, but removal of the seed oil is not as complete as with Swedish tube method (5% vs 3% remaining, respectively) (McGregor, unpublished).

Removal of moisture from vegetative samples reduces bulk, effectively increasing the concentration of glucosinolates. Moisture is usually removed after aqueous or aqueous–methanol extraction. A typical procedure (33) macerates fresh samples in boiling methanol, further extracts with 70% methanol, and then concentrates the extract under reduced pressure. Heaney and Fenwick (41), noting that vegetative samples if quickly frozen would retain their glucosinolates intact, varied this approach by first freezing samples (−40°C), grinding in the frozen state (−20°C), and then extracting with boiling methanol. Freeze-drying has also been used to reduce moisture content (42). Freezing facilitates handling of large numbers of samples because samples can be accumulated and stored in

the frozen or freeze-dried state. But great care must be taken to ensure that the samples do not thaw or otherwise contact moisture, because myrosinase will still be active.

Inactivation of Myrosinase and Cofactors

The co-existence of myrosinase and cofactors in glucosinolate-containing plants necessitates inactivation by heat treatment if glucosinolates are to be extracted intact, or if enzymatic hydrolysis is to be carried out under controlled conditions. Temperatures of 70 to 110°C are usually high enough to inactivate myrosinase (25) but low enough to prevent thermal decomposition (33).

Heating before cell disruption, using a forced-air oven (43, 44), microwave radiation (45–47), or short immersion in boiling water (45) has been effective in inactivating myrosinase and cofactors in seed. However, the seed must have a moisture content of at least 8–10%. If less, the moisture content of sound seed should be increased by the addition of water before heat treatment. Alternatively, seed with a moisture content of less than 4% may be oil-extracted without risk of myrosinase hydrolysis (43). Heat treatment is subsequently applied to the meal. Myrosinase in seed meals has been conveniently inactivated by the addition of boiling water (48, 49), buffer (43, 50), water–methanol (51), or water–ethanol mixtures (52). An advantage of boiling water or water–alcohol for the inactivation of myrosinase is that their use effectively limits the temperature of inactivation and hence prevents thermal decomposition. The polar nature of these solvents may also assist in inactivating myrosinase cofactors.

Inactivation of myrosinase and cofactors in vegetative material is usually facilitated by using methanol (41, 42, 53) or water–methanol mixtures (54–56) while simultaneously extracting the glucosinolates. Microwave treatment has also been advantageous in this respect (Wilkinson and Fenwick, unpublished observation).

Heating to inactivate endogenous myrosinase and cofactors, then adding a myrosinase isolate and hydrolyzing at neutral pH has been used to control the nature and number of hydrolysis products. Only isothiocyanates and products derived from them, oxazolidinethiones or thiocyanate ion, are produced. This approach has been particularly useful for indirectly determining glucosinolate content (53, 57). Yellow/white mustard is usually chosen as the source of myrosinase preparation because of the relatively high enzymatic activity (25) and absence of epithiospecifier protein (37). The myrosinase is prepared by aqueous acetone (58) or aqueous ethanol extraction (59, 60). Extracting with polar solvents presumably frees the isolate of nitrile- and thiocyanate-producing cofactors, if indeed they exist in yellow/white mustard seed.

Identification

Since the pioneering work of Gadamer at the end of the last century (61) and up until very recently, qualitative analysis of glucosinolates has been confined almost entirely to identification of their enzymatically, and chemically, produced breakdown products. Compounds most readily used for structural purposes have been the isothiocyanates (often after conversion to their thiourea derivatives), substituted oxazolidinethiones, and 1,3-oxazinethiones which are formed during enzyme hydrolysis. Organic acids produced by acid hydrolysis have also been used. In addition to being readily purified, separated, and chemically analyzed, the structures of these compounds have often been confirmed by chemical synthesis (2).

"Identification of glucosinolate structure based solely on analysis of enzymatic or chemical products depends on the tacit assumption that all glucosinolates possess the same general skeleton and differ only in the chemical structure of the side chain."

Identification of glucosinolate structure based solely on analysis of enzymatic or chemical products depends on the tacit assumption that all glucosinolates possess the same general skeleton and differ only in the chemical structure of the side chain. Linscheid and co-workers' (5) recent identification of a glucosinolate differing in general structure by possessing a 6-sinapoyl-

thioglucose moiety clearly indicates the need for caution with this approach. Certainly the nature of the carbohydrate produced under chemical or enzymatic conditions needs confirming. Ideally, structural assignments proposed on the basis of breakdown products should be confirmed by study of the intact glucosinolate, or desulfoglucosinolate obtained by treating glucosinolates with sulfatase (aryl-sulfate sulfohydrolase, EC 3.1.6.1).

The modern phase of glucosinolate study, which may be considered to have begun some 3 decades ago with the research of O. E. Schultz and A. Kjaer, has coincided with the development of chromatographic techniques. Paper, thin layer, and gas chromatography and later gas chromatography–mass spectrometry and high performance liquid chromatography have all been used to identify enzymatic or chemical products of glucosinolates (12, 62, 63). More recently, all of these techniques have been used to identify intact glucosinolates or derivatives thereof.

Enzymatic and Chemical Products

(a) *Paper Chromatography.*—Paper chromatography has been most extensively used to separate thioureas, formed by reaction of the enzymatically released isothiocyanates with ammonia. Kjaer and Rubinstein (64) first described the use of paper chromatography for the separation of thioureas. The potential of the technique was immediately obvious and formed the basis of much of the prolific work of the Copenhagen group. Water-saturated chloroform was the most suitable system for ascending chromatography, providing that precautions were taken to ensure equilibrium in the developing chamber. The same solvent system was later used by Josefsson (16) for descending chromatography. Thioureas were detected using Grote's reagent, or starch followed by iodine azide (16, 64).

Hydrophilic thioureas, poorly resolved with water-saturated chloroform, were effectively separated by Ettlinger and Thompson (65) using *n*-butanol–toluene–water (3 + 1 + 1). With this and other solvent systems, *n*-butanol–toluene–water (1 + 5 + 2), toluene–acetic acid–water (5 + 2 + 4) and ethanol–benzene–water (1 + 5 + 2), a major screening program was carried out on glucosinolate-containing seeds (65). Rodman and others (62, 66–69) used paper chromatography extensively for the examination of thioureas as part of elegant chemotaxonomic studies. Rodman claimed that this technique was particularly useful for the investigation of plants producing oxazolidinethiones and isothiocyanates possessing ω-methylsulfinylalkyl or ω-methylsulfonylalkyl side chains. Comprehensive R_f values of thioureas have been published (62, 65).

Paper chromatography has also been applied to the separation and identification of organic acids which may be formed from glucosinolates upon acid hydrolysis (55, 63). These compounds were detected with aniline-xylose reagent.

(b) *Thin Layer Chromatography.*—Unlike paper chromatography, thin layer chromatography has not been extensively used for the investigation of glucosinolate breakdown products. Wagner and co-workers (70) used ethyl acetate–chloroform–water (3 + 3 + 4) to separate thioureas on silica gel. Josefsson (16) separated the same derivatives with chloroform–ethanol (9 + 1). Reagents used for detection included ferricyanide/ferric chloride (70), Grote's reagent, or starch followed by iodine azide (16). Josefsson claimed that the detection limit for thioureas was much lower than on paper (16). Rodman (62) also recommended the use of this technique for investigation of thioureas and oxazolidinethiones. In studies on indole glucosinolates, Gmelin (71) reported the separation of products from enzymatic and chemical treatment of 3-indolylmethyl glucosinolate (glucobrassicin), using chloroform–ethanol (99 + 1). These products were detected with Ehrlich's reagent.

(c) *Gas Chromatography.*—The first reported use of gas chromatography for the separation of isothiocyanates appears to be that of Kjaer and Jart (72). Methyl, ethyl, propyl, isopropyl, and isopentyl isothiocyanates were separated, clearly identifying the potential of the technique for separation, measurement, and identification of volatile isothiocyanates of natural origin. Jart (73) published gas chromatographic data on a number of isothiocyanates. Kjaer and Friis (74) applied gas chromatography to examination of glucosinolate derived volatiles from *Putranjiva roxburghii* Wall. Kjaer and Thomsen (75) used gas chromatography to isolate 5-oxoheptyl isothiocyanate, the structure of which was proven with the aid of mass spectrometry.

In recent years, gas chromatography has been extensively used for the investigation of glucosinolate breakdown products. A variety of columns, including Carbowax 20M (36, 76, 77), EGSS-X (53, 69), Apiezon L (36, 53), FFAP (44), and OV-17 and OV-1 (56) have been used with temperature programming to separate isothiocyanates (36, 53, 56, 69, 76, 77), oxazoli-

dinethiones (56), nitriles (53, 77), epithionitriles (36), epithioalkanes (76), and trimethylsilyl derivatives of organic acids (56). In many instances, gas chromatography has been linked with mass spectrometry.

It is important to emphasize that identification of breakdown products by gas chromatography should include comparison with standard compounds. Two or more columns of differing polarity, sufficient to provide adequate differences in elution times, should be used. Such was the case in studies by VanEtten and co-workers (57, 78, 79).

(d) *Gas Chromatography–Mass Spectrometry.*—Gas chromatography-mass spectrometry has been extensively employed in the investigation of glucosinolate breakdown products, due in large part to interest in the volatile compounds which contribute to the flavor of glucosinolate-containing plants and seeds (2, 80). In 1961, the mass spectrum of allyl isothiocyanate was reported in conjunction with a study of the flavor constituents of cabbage by Bailey and co-workers (81). In 1963, Kjaer and co-workers (82) published detailed mass spectra of several isothiocyanates. Fragments produced by these isothiocyanates have subsequently been rationalized by Bach and co-workers (83). More recently, a comprehensive compilation of mass spectra of various types of glucosinolate decomposition products (nitriles, isothiocyanates, and oxazolidinethiones) has been published (84). Other less comprehensive listings of relevant mass spectra have also been produced (76, 85, 86).

Combined gas chromatography-mass spectrometry has been used for the identification of 1-cyanoepithioalkanes (36, 87), autolysis products of glucosinolates (88), nitriles (89), and isothiocyanates (57, 76, 79). Grob and Matile (24) recently used capillary gas chromatography-mass spectrometry for an analysis of the volatiles of horseradish and reported the presence of as many as 30 glucosinolates.

When gas chromatography-mass spectrometry is applied to investigate the nature of glucosinolate breakdown products, it is recommended that, where possible, confirmation of structural assignment be made by using authentic standards on columns of differing polarity.

(e) *High Performance Liquid Chromatography.*—Possibly because of the volatility of most glucosinolate decomposition products, and thus their amenability to gas chromatography, high performance liquid chromatography has not been extensively applied to the investigation of these compounds. Maheshwari and co-workers (90, 91) described a method for the separation of organic isothiocyanates and oxazolidinethione of rapeseed by using high performance liquid chromatography and another method has been reported for the determination of oxazolidinethiones in milk by Benns and co-workers (92). Harris and co-workers (93) used high performance liquid chromatography to analyze for oxazolidinethiones, avoiding losses encountered with gas chromatography thought to result from thermal decomposition. High performance liquid chromatography has been valuable in the separation and purification of other higher molecular weight isothiocyanates, such as ω-methylsulfinylalkyl and ω-methylsulfonylalkyl isothiocyanates, which are also difficult to study by gas chromatography (38).

"High performance liquid chromatography potentially affords the advantage of direct determination of glucosinolates, but a limitation to its widespread use is the restricted means available for detection of compounds eluted from the columns."

A limitation to widespread use of high performance liquid chromatography is the restricted means available for detection of compounds eluted from the columns. Henning (94) has recently shown that many glucosinolates and their breakdown products can be separated by high performance liquid chromatography using a single run on one column. However, the technique is limited by the fact that some products, notably thiocyanates and nitriles, are not detectable spectrophotometrically. Mullin (95) has also noted that problems can occur as a result of reactivity between isothiocyanates and mobile phases containing methanol and has emphasized that use of this solvent should be avoided.

High performance liquid chromatography should be of use in the examination of indole, phenolic, and side chain *O*-glycosylated glucosinolates. It is expected that this technique will find increasing application in the future, for both qualitative and quantitative analysis and in the preparation of purified glucosinolates and their breakdown products.

Glucosinolates

(a) *Paper Chromatography.*—Kjaer (12) has pointed out that while the use of paper chromatography has helped to establish the glucosinolate patterns of numerous plant species, its application to obtain unambiguous identification of glucosinolates is limited because of the relatively narrow range of R_f values found for glucosinolates. Such caution remains justified. The technique is best used in combination with others.

Schultz and Gmelin (96, 97) and Schultz and Wagner (98) developed methods for the separation of glucosinolates from crude extracts of plant material by using paper chromatography. Compounds were visualized with 10% copper sulfate or ammoniacal silver nitrate solutions. Solvent systems included *n*-butanol–acetic acid–water, in various proportions, and iso-butanol–acetic acid–water (4 + 1 + 5). Kjaer and Gmelin (99) reported the use of pyridine–amyl alcohol–water (35 + 30 + 30) and *n*-butanol–ethanol–water (4 + 1 + 4, upper phase) in a comprehensive screening of glucosinolates in *Alyssum* species. The latter system has also been used by others (16, 55). Underhill and Kirkland (48) separated glucosinolates with *n*-butanol–acetic acid–water (20 + 5 + 9).

Olsen and Søørensen (55, 56) used *n*-butanol–acetic acid–water (12 + 3 + 5) and *n*-butanol–pyridine–water (6 + 4 + 3) in studies on glucosinolates possessing *O*-glycosylated side chains. These workers emphasized the importance of sample cleanup before chromatography, advocating the use of ion exchange techniques for this purpose (63).

Rakow and co-workers (100) recently reported R_f values of a number of desulfoglucosinolates, using *n*-butanol–acetic acid–water (4 + 1 + 2). Ammoniacal silver nitrate was used as a general spray reagent. A number of other reagents were cited as suitable for detecting specific glucosinolates.

(b) *High Voltage Electrophoresis and Isotacophoresis.*—High voltage paper electrophoresis for qualitative or preparative studies of glucosinolates has been pioneered recently by Danish workers (63). Three different buffer systems have been used (pH 1.9, 3.6, and 6.5). Preparative electrophoresis of glucosinolates under the most acid conditions afforded their purification from contaminants present in plant extract, but some decomposition occurred (55). This was reduced if the papers were allowed to dry in an atmosphere of ammonia.

Using isotacophoresis, Klein (101) has been able to separate and quantitate allyl glucosinolate from brown (*B. juncea*) and black mustard and 4-hydroxybenzyl glucosinolate from yellow/white mustard.

(c) *Thin Layer Chromatography.*—Although paper chromatography has proven much more useful, glucosinolates have been separated by thin layer chromatography. For thin layer chromatography on silica gel, *n*-butanol–*n*-propanol–acetic acid–water (3 + 1 + 1 + 1) was originally used as a solvent system, with detection accomplished by spraying with trichloroacetic acid in chloroform followed by ferricyanide/ferric chloride (70) or alkaline silver nitrate (48). Matsuo (102) described improved chromatographic behavior on silica gel with various solvent systems, but found that some glucosinolates decomposed when pyridine was included in the developing mixture. Glucosinolates were detected by treatment with iodine vapor, or under ultraviolet (254 nm) light.

Recently, Rakow and co-workers (100) separated desulfoglucosinolates on silica gel, using *n*-butanol–acetic acid–water (4 + 1 + 2) and *n*-butanol–*n*-propanol–acetic acid–water (3 + 1 + 1 + 1) and listed R_f values. The compounds were visualized with thymol-sulfuric acid.

(d) *Gas Chromatography.*—Gas chromatography of glucosinolates has been made possible by the application of trimethylsilyl derivatization, which renders the glucosinolates sufficiently volatile for separation. When glucosinolates are derivatized, they undergo desulfation (48), so it is actually the trimethylsilylated desulfoglucosinolates which are separated. Glucosinolates and desulfoglucosinolates can be differentiated if treated with myrosinase before derivatization. While glucosinolates are hydrolyzed by this enzyme, desulfoglucosinolates are apparently not affected (4). It has been shown that the desulfoglucosinolate is an intermediate in glucosinolate biosynthesis (103); therefore, desulfoglucosinolates could occur in plants along with the glucosinolates.

Gas chromatography of trimethylsilylated desulfoglucosinolates to determine glucosinolate composition was first described by Underhill and

Kirkland (48) in 1971. Glucosinolates were extracted with boiling water and derivatized, and separations were carried out by isothermal chromatography. Substantial improvements have been subsequently made in this method.

Ion exchange purification of glucosinolate extracts to remove carbohydrates and other impurities before derivatization was introduced by Thies (49, 104) to increase sensitivity. Following the binding of glucosinolates onto a column of Sephadex A-25, neutral substances, particularly sucrose, were removed with water washing. The purified glucosinolates were then removed with potassium sulfate. Difficulty in removing the potassium sulfate from the eluted glucosinolates before derivatization led to the use of pyridine acetate (105) and later imidazole formate (106) for elution. The latter 2 eluants also permitted regeneration of the ion exchange column. In addition to being able to determine as little as 10 μmoles of glucosinolate per gram of seed meal, the method could also be applied to vegetative samples which contained large amounts of carbohydrate.

In addition to ion exchange purification, Thies (106–108) introduced desulfation before derivatization. Thies reported that sulfate released from the glucosinolates during derivatization interfered with subsequent gas chromatographic separation (106, 107). Desulfation was elegantly carried out on the ion exchange column, using a commercially available sulfatase isolated from an edible snail (*Helix pomatia*). Free sulfate in the glucosinolate extract, which could inhibit the sulfatase, was precipitated by the addition of barium acetate/lead acetate and removed by centrifugation before addition of the extract to the ion exchange column. When vegetative extracts were analyzed, weak acids, including phenolcarbonic acids and phenols, were removed by washing the column with dilute acetic acid followed by water to prevent inhibition of the sulfatase (108). The sulfatase was then added directly to the column and the desulfoglucosinolates were eluted with water. Heaney and Fenwick (109) have subsequently found that desulfation before derivatization is essential to successfully derivatize indole glucosinolates.

A variety of trimethylsilyl reagents has been used to carry out the derivatization, including hexamethyldisilazane and trimethylchlorosilane (48), *N,O*-bis-(trimethylsilyl)trifluoroacetamide (56), and *N*-methyl-*N*-trimethylsilylheptafluorobutyramide in combination with trimethylchlorosilane (49, 109). The latter mixture has been preferred because it minimizes detector contamination. As a strong donor it also promotes complete derivatization, avoiding the formation of multiple peaks. Multiple peaks were a problem in early studies, particularly when short derivatization times or low derivatization temperatures were used (48, 110). Although Thies (107) achieved derivatization of alkenyl and hydroxyalkenyl glucosinolates from rapeseed in 1.5 h at room temperature or by heating at 80°C for 15 min, Heaney and Fenwick (111) found that a higher temperature of 120°C was required for efficient derivatization of indole glucosinolates.

Because of possible decomposition of trimethylsilyl derivatives on metal columns, the use of glass columns has generally been preferred. Separations were initially made by isothermal chromatography on packed columns of SE-52 (48, 49) but OV-7, originally introduced by Persson (52), has been commonly used since Thies (104), in a comparison of OV-3, OV-7, OV-17, OV-25, OV-101, OV-210, different UCON types, and different Carbowax types, found it to give the best separation of the alkenyl and hydroxyalkenyl glucosinolates from rapeseed. Heaney and Fenwick (109) introduced temperature programming with packed columns. They found temperature programming to be essential for the separation and detection of the indole glucosinolates. Hiltunen and co-workers (112) have separated the alkenyl and hydroxyalkenyl glucosinolates from rapeseed using an SE-52 capillary column and temperature programmed chromatography. Recently, capillary column chromatography with an SE-54 column has been used to separate the trimethylsilyl derivatives of 4-hydroxy-3-indolylmethyl and 3-indolylmethyl glucosinolates (113).

Heaney and Fenwick (41, 109) used gas chromatography of trimethylsilyl derivatives to obtain good resolution of the individual glucosinolates of *Brassica* vegetables. However, 3-methylsulfinylpropyl glucosinolate (glucoiberin) was found to be atypical in that more than one compound was evident after derivatization. Recent work using combined gas chromatography and ammonia chemical ionization mass spectrometry has indicated that one of these multiple peaks results from silylation of the sulfinyl oxygen (Eagles, Heaney, and Fenwick, unpublished). It is yet unclear whether this behavior is related to the silylation reagents used by these workers, but variation of the derivatization conditions had no effect. Thies (personal communication) has not found this problem to occur with 4-methylsulfinylbutyl glucosinolate

(glucoraphanin), using similar techniques. This problem is of relatively little importance to the glucosinolate analysis of seeds since they contain only trace amounts of 3-methylsulfinylpropyl glucosinolate (glucoiberin). However, it is known that this glucosinolate occurs widely in the vegetative tissues of *Brassica* species, where it can be the predominant glucosinolate (2, 53). The finding that the corresponding methylsulfonyl or thiomethyl glucosinolates are derivatized without problems has led to the suggestion (Gmelin, unpublished) that methylsulfinyl glucosinolates might be oxidized or reduced before analysis. No detailed studies of this problem have been published yet, but it represents a very real limitation to the gas chromatography of trimethylsilyl derivatives of glucosinolates.

(e) *Gas Chromatography–Mass Spectrometry.*— Olsson and co-workers (114), in an examination of the mass spectra of desulfoglucosinolates using electron impact, found that minor fragmentation could yield some structurally useful information, although the major fragments were of little use for structural purposes. Subsequently, mass spectra of both intact glucosinolates and desulfoglucosinolates have been reported using electron impact (115) or chemical ionization with methane, isobutane, or, most usefully, ammonia (116). Structurally significant ions containing the side chain (e.g., R^+, RCN^+, and $RCHN^+OH$) were noted. More recently, Eagles and co-workers (117) have applied ammonia gas chemical ionization to the trimethylsilylated desulfoglucosinolates described above. A considerable improvement in the amount of structurally useful fragmentation was evident compared with that reported from earlier electron impact studies (114). The technique has proved amenable to both single compounds and mixtures. Christensen and co-workers (118) have used isobutane gas chemical ionization of trimethylsilylated desulfoglucosinolates with similar results. Gas chromatography–chemical ionization mass spectrometry, with single ion monitoring, has been employed by Fenwick and co-workers (119) to investigate the extent of wild mustard (*B. kaber* (DC) L. C. Wheeler), also known as charlock (*S. arvensis* L.), contamination of rapeseed meal. Linscheid and co-workers (5) have described the use of field desorption mass spectrometry in the course of structure elucidation of 6-sinapolythiogluco-4-methylsulfinylbutenyl glucosinolate.

The lack of volatility on the part of glucosinolates and desulfoglucosinolates suggested that they might be examined with advantage by the recently described technique of fast atom bombardment (120, 121). Positive ion fast atom bombardment yielded mass spectra characterized by abundant protonated and cationized molecular ions (corresponding to the intact salts). Relatively little fragmentation occurs but since many glucosinolates have unique molecular weights this is not necessarily a severe disadvantage. In the negative ion mode, fast atom bombardment produces an abundant molecular ion (of the glucosinolate anion). This proved especially advantageous in the analysis of crude plant extracts and mixtures of purified glucosinolates. Good agreement was also noted between the results of negative ion fast atom bombardment and combined gas chromatography–mass spectrometric techniques for the analysis of plant extracts (121). Such techniques may thus have considerable potential for the investigation of glucosinolate composition.

(f) *High Performance Liquid Chromatography.*— High performance liquid chromatography potentially affords the advantage of direct determination of glucosinolates. The first successful application of the technique was described by Helboe and co-workers (122). Glucosinolates were first purified by ion exchange, and then separated by reverse phase ion-pairing chromatography using a C_{18} column of Nucleosil 5 (5 μm), phosphate buffer at pH 7 modified with 70% methanol as the mobile phase, and tetraheptylammonium or tetraoctylammonium bromide as the source of counter ion. Capacity factors (k') for a number of glucosinolates isolated from natural sources were determined (63, 122). Although the method offers potential for both qualitative and preparative purposes by avoiding acidic and basic conditions which can lead to glucosinolate decomposition, some glucosinolates were only partially separated while others were not separated at all.

Minchinton and co-workers (113) have recently described a method for separating desulfoglucosinolates. Reverse phase chromatography on a C_{18} column of Zorbax ODS (5–6 μm), using gradient elution with acetonitrile, separated most of the glucosinolates found in *Brassica* species. By avoiding the use of buffer solutions and ion-pairing reagents, glucosinolates could be collected in a pure form suitable for identification by mass spectrometry. With the aid of this method, 2 new glucosinolates were separated and identified, 4-hydroxy-3-indolylmethyl glucosinolate from the seed of cabbage and rapeseed (123) and 4-methoxy-3-indolylmethyl

glucosinolate from the edible parts of brussels sprouts, cabbage, and cauliflower (124).

(g) *Nuclear Magnetic Resonance.*—As in other branches of natural product chemistry, the advent and application of nuclear magnetic resonance (NMR) spectroscopy has facilitated structural analysis. Kjaer and co-workers (125, 126) have published ^{1}H NMR spectra of acetylated glucosinolates. Underhill and Kirkland (48) have published ^{1}H NMR spectra of trimethylsilyl glucosinolates and desulfoglucosinolates, but with little detail. The first reports of ^{13}C NMR spectra of glucosinolates appear to be those of Olsson et al. (114) and Friis et al. (127).

In the course of recent studies, Olsen and Sørensen (55, 56) used both ^{1}H and ^{13}C NMR for the identification of intact glucosinolates. The same authors also reported ^{13}C chemical shifts for a range of glucosinolate pyridinium salts (63). Linscheid and co-workers (5) published the ^{1}H and ^{13}C NMR spectra of 6-sinapolythiogluco-4-methylsulfinylbutenyl glucosinolate and also compared the ^{1}H NMR spectrum of this compound in d_6-dimethylsulfoxide and d_4-methanol. A detailed analysis of ^{1}H and ^{13}C spectra of glucosinolates and desulfoglucosinolates has recently been compiled (Cox, Hanley, Belton, and Fenwick, manuscript submitted).

Quantitation

The contribution made by glucosinolate breakdown products to the flavor or biological effects, and thus the value, of agricultural crops and commodities has over the years stimulated considerable interest in methods for determining their glucosinolate content. A wide diversity of methods have been developed. These included both indirect methods, in which one or another of the enzymatically released products are measured, and direct methods in which intact glucosinolates or derivatives thereof are measured. However, few of the methods developed to date are universally applicable to all, or even most, of the glucosinolates known to exist in nature. Most methods have been developed for specific agricultural crops or commodities and their particular glucosinolate compositions.

As instrumentation, and knowledge of the diversity, distribution, and chemistry of glucosinolates have improved, analytical methodology for determining glucosinolate content has evolved and improved. To better understand this evolution and improvement, it is convenient to present methods in relation to the specific crops, commodities, and their particular glucosinolates, for which they were originally developed and for which they are particularly suited.

Enzymatic and Chemical Products

Until recently, analysis for glucosinolate content of agricultural crops or their commodities was only possible by indirect measurement of one or another of the enzymatically or chemically released products. Most of the products of myrosinase hydrolysis, including isothiocyanates, oxazolidinethiones, nitriles, thiocyanate ion, sulfate, and glucose, have been measured to determine the glucosinolate content of various crops and commodities.

(a) *Isothiocyanates.*—Many of the methods available for determining glucosinolate content before 1950 were originally developed to analyze for allyl isothiocyanate produced from allyl glucosinolate in black mustard seed (128). Common to all methods is the enzyme hydrolysis of the glucosinolate to produce the isothiocyanate followed by reaction with ammonia to produce the substituted thiourea. The thiourea is preferred because it was found to enter more readily than the isothiocyanate into reactions that could be used for quantitative measurement. Three methods described in older literature which use this approach involve gravimetric, argentimetric, and iodometric procedures.

The gravimetric method is the oldest, originally devised in 1871 by Fluckiger and revised by Hager in 1883 (129). Following enzyme hydrolysis of the glucosinolate, the isothiocyanate was steam-distilled, reacted with ammonia to form the substituted thiourea, then quantitated by weighing. The method was adopted by the 2nd edition of the German Pharmacopoeia in 1882 and retained in the 3rd edition of 1890, but replaced in the 4th edition of 1900 because it was considered to be too long and laborious (129). The gravimetric approach was replaced by the argentimetric method. Following enzyme hydrolysis of the glucosinolate to produce the isothiocyanate, steam distillation and reaction with ammonia, the thiourea was decomposed by reaction with silver ion. In the original method proposed by Dietrich in 1886 the insoluble silver sulfide produced was collected and quantitatively determined by weighing (129). Later, Gadamer (130, 131) transformed it into a volumetric method: The silver sulfide was filtered off and the unreacted silver was back-titrated with thiocyanate.

The argentimetric method is still a recommended method for analysis of mustard. The

AOAC listed it essentially unchanged from the original proposal of Gadamer (132, 133). In the most recent AOAC method (133), ground mustard (species not specified) is incubated in 5% ethanol for 1½ h at 37°C. Enzymatically released isothiocyanate is then steam-distilled into ammonium hydroxide. Silver nitrate is added and the solution is left overnight at ambient temperature for decomposition of the thiourea. Following heating and filtering to agglomerate and remove the silver sulfide, the unreacted silver is back-titrated with ammonium thiocyanate, using ferric ammonium sulfate as indicator.

Early variations of the argentimetric method concerned optimal conditions for reaction of the isothiocyanate with ammonia. Conditions used include aqueous or more often alcoholic ammonium hydroxide, and reaction times and temperatures of ambient for up to 48 h, to warming to about 50°C for as long as 15 h (128). More recently, variations have concerned the time and temperature of the reaction with silver to decompose the thiourea. Reaction for 12 h (134) and 4 h (135) at ambient temperature, and 1 h at 80°C (136, 137) have been used.

"The argentimetric method is still a recommended method for analysis of mustard. The AOAC listed it essentially unchanged from the original proposal of Gadamer. With both the gravimetric and argentimetric approaches, usually no attention was paid to control of the myrosinase step."

With both the gravimetric and argentimetric approaches, usually no attention was paid to control of the myrosinase step. However, one method, the official method of the American Spice Trade Association (135), does recommend inactivation of endogenous myrosinase, and any cofactors present, and addition of an external source of enzyme. Higher values compared with the AOAC method (135) were obtained, presumably because the formation of hydrolysis products other than the isothiocyanate was reduced. These differences emphasize the importance of controlling myrosinase hydrolysis.

Morvillez and Messemaeker (138, 139) originally proposed the iodometric procedure as a more rapid method than the argentimetric method for determining allyl isothiocyanate. Following enzyme hydrolysis, the isothiocyanate was steam-distilled and reacted with ammonia to form the thiourea, then the reaction mixture was acidified, iodine was added, and the extent of decoloration resulting from reaction of the iodine was measured. A later modification by Meesemaeker and Boivin (140) facilitated the analysis by eliminating the need to isolate the isothiocyanate by steam distillation. Instead, the reaction mixture was clarified by the addition of lead acetate before colorimetric analysis. As with the argentimetric methods, the need for controlled enzymatic hydrolysis conditions was not appreciated.

In the early 1950s, Kjaer and co-workers (141) took advantage of the intense ultraviolet absorption of substituted thioureas of isothiocyanates, including allyl, to develop a spectrophotometric method for quantitation. Ground seed was first added to a mixture of hot petroleum ether and ethanol and refluxed to inactivate the endogenous enzymes and partially extract the oil. Oil extraction was completed by further refluxing with petroleum ether and ethanol. A myrosinase isolate from yellow/white mustard was added to the oil-extracted meal and the mixture was incubated. The released isothiocyanates were then steam-distilled into ammonia to form thioureas. The clear solution containing the thioureas was then evaporated to dryness and diluted to volume with ethanol, and the absorbance was measured at 220, 240, and 260 nm. A corrected extinction coefficient for the peak at 240 nm was calculated by adjusting for background absorption, using the absorptions at 220 and 260 nm. Nagashima and Nakagawa (142) used only one wavelength, 237 nm, to measure allyl isothiocyanate. Langer and Gschwendtova (143) carried out hydrolysis at controlled pH and extracted with ether rather than steam distilling to obtain the isothiocyanate.

Analysis for allyl isothiocyanate has been facilitated by reaction with amines rather than ammonia. Chikkaputtaiah and co-workers (144) proposed a method for the specific analysis of

allyl isothiocyanate from black mustard in the presence of cyanides, thiocyanates, or organic sulfides. Comminuted mustard seed was mixed with water and incubated to hydrolyze the glucosinolate. The released isothiocyanate was steam-distilled into ethanol and reacted with either piperidine or pyrrolidine; then the unreacted piperidine, or pyrrolidine, was determined by titration with acid. The method is reputed to be relatively rapid and the reagents are less expensive than for the argentimetric method. A modification of the method was applied to the determination of 4-hydroxybenzyl glucosinolate in yellow/white mustard by Shankaranarayana and co-workers (145). The isothiocyanate was reacted with piperidine, then the excess was back-titrated with sulfuric acid. The method compared favorably with the iodometric method and was reputed to be simpler.

Recently, Devani and co-workers (146) have proposed a colorimetric method for determining allyl isothiocyanate. The isothiocyanate was reacted with sulfanilamide to form the corresponding thiourea, which was then allowed to react with 2,3-dichloro-1,4-naphthoquinone in the presence of ammonia before measuring the absorbance at 540 nm. The absence of any requirement for steam distillation in this method may be considered a particular advantage.

Degradation reactions have also been used to measure allyl isothiocyanate. Maier and Diemair (147) suggested reacting allyl isothiocyanate in foodstuffs with *N,N*-dimethyl-*p*-phenylene-diamine to form a colored complex. Shankaranarayana and co-workers (148) proposed measuring the allyl isothiocyanate released from black mustard by oxidation with chloramine-T in dilute sulfuric acid followed by addition of iodide and titration of the excess chloramine-T with thiosulfate. Inexpensive and stable reagents, speed of analysis relative to the argentimetric method, and sensitivity were cited as assets (148, 149).

Attempts have been made to specifically analyze for allyl isothiocyanate by using paper chromatography to isolate the thiourea (141, 143). This approach is, however, tedious and time consuming. Application of the gas chromatography method of Youngs and Wetter (44) (see below) has proven more appropriate (150). A modification of Youngs and Wetter gas chromatography method has been adopted by AOAC (133, 151). However it suffers from use of an external rather than an internal standard, and failure to control the conditions of myrosinase hydrolysis.

High performance liquid chromatography can be used to rapidly and specifically quantitate minute amounts of allyl isothiocyanate. Maheshwari and co-workers (90, 91) have been able to detect as little as 0.2 μg allyl isothiocyanate per mL extract. But care must be exercised to avoid use of alcohols for extraction or in the mobile phase; Mullin (95) has shown that these can react with isothiocyanates.

Gravimetric, argentimetric, and iodometric methods, originally developed to measure allyl isothiocyanate produced from black mustard seed, have also been applied to the seed of brown mustard and rapeseed. But by the 1950s, lack of agreement between methods, attributed to differences in glucosinolate composition, had led to doubt as to their reliability when thus employed (129, 152). In addition to allyl glucosinolate, brown mustard of Asian origin contains appreciable amounts of 3-butenyl glucosinolate (gluconapin) (153). Rapeseed contains only trace amounts of allyl glucosinolate with both 3-butenyl and 4-pentenyl (glucobrassicanapin) as major glucosinolates. Wetter (154) improved the reproducibility of the argentimetric method when applied to rapeseed meal by controlling the pH of hydrolysis. But the requirement for a relatively large sample, and the tedious and time consuming nature of the steam distillation and titration, led to a search for better methods.

Youngs and Wetter (44) employed gas chromatography to facilitate the quantitation of 3-butenyl and 4-pentenyl glucosinolates in rapeseed. Isothiocyanates released during hydrolysis were simultaneously extracted into methylene chloride. Sample size was reduced from 10 g to 10 mg. Quantitation was achieved by adding butyl isothiocyanate as an internal standard to the methylene chloride. Rigolier (150) demonstrated that the method could also be used to measure allyl isothiocyanate and applied the method to black and brown mustard.

Wetter and Youngs (155) further improved the analysis of 3-butenyl and 4-pentenyl glucosinolates in rapeseed by subsequently employing ultraviolet measurement of the thioureas. Following extraction of the enzymatically released isothiocyanates with methylene chloride, an aliquot was transferred to ethanolic ammonia to form the thioureas. The method increased throughput because it lent itself to batch-type operation.

More recently, Maheshwari and co-workers (90, 91) have used high performance liquid chromatography to quantitate 3-butenyl and

4-pentenyl isothiocyanates. Gas chromatography of commercial allyl isothiocyanate and rapeseed meals containing 3-butenyl and 4-pentenyl isothiocyanates were used to calibrate the high performance liquid chromatography (91).

(b) *Thiocyanate Ion.*—Most glucosinolates give rise to stable isothiocyanates which may be isolated and measured. Notable exceptions are the indole glucosinolates, 3-indolylmethyl- and 1-methoxy-3-indolylmethyl glucosinolate (neoglucobrassicin), and 4-hydroxybenzyl glucosinolate whose isothiocyanates are unstable and break down under neutral or alkaline conditions to release inorganic thiocyanate ion. Indole glucosinolates appear to have an ubiquitous distribution among the brassicas. They have been found in the vegetative parts of all members of the genus investigated to date. Recently, they have also been reported to be present in substantial amounts in the seed (23, 109). 4-Hydroxybenzyl glucosinolate is the major glucosinolate in the seed of yellow/white and wild mustard.

Ashworth (128) extensively reviewed the analytical chemistry of inorganic thiocyanates. He classified most methods into 2 groups: those based on conversion to poorly soluble and/or colored thiocyanates, and those in which the thiocyanate undergoes oxidation. Both approaches have been used to determine indole and 4-hydroxybenzyl glucosinolate content of agricultural products.

Reaction of thiocyanate ion with ferric chloride or ferric nitrate to produce an intensely red ferric thiocyanate complex has been widely used to measure the indole glucosinolate (20, 54, 156, 157) and 4-hydroxybenzyl (50, 65, 158) content of brassicas. Recently, analysis has been facilitated by automation with a continuous flow analyzer (157).

In some studies, the method has been limited by interfering substances that react with ferric ion to give background color and artificially high values, or by precipitation of some of the thiocyanate which results in low values (159). To overcome these difficulties, Butcher and coworkers (160) isolated indole glucosinolates by paper chromatography prior to myrosinase hydrolysis. Paxman and Hill (20) treated extracts with charcoal to reduce background color. Johnston and Jones (156) added a little mercuric chloride to destroy the ferric thiocyanate complex and subtracted the background measurement from the original value.

McGregor (unpublished) has found that 4-hydroxybenzyl glucosinolate can be determined in the presence of indole glucosinolates by selectively extracting 4-hydroxybenzyl isothiocyanate into methylene chloride. Myrosinase hydrolysis was carried out at pH 4 while simultaneously extracting with methylene chloride. After an aliquot was transferred to water, the methylene chloride was driven off by gentle heating and swirling; thiocyanate ion formed on treatment with alkali was reacted to form a ferrithiocyanate complex.

Although inactivation of endogenous myrosinase and cofactors plus buffering of the hydrolysis pH has not always been advocated for the determination of those glucosinolates which give rise to thiocyanate ion, this may not be a serious oversight. Srivastava and Hill (161) reported that about the same amount of thiocyanate ion was formed when rapeseed meal was autolyzed as when the meal was first heated and then hydrolyzed at pH 7 with added myrosinase, even though the alkenyl and hydroxyalkenyl glucosinolates gave rise to isothiocyanates and nitriles plus oxazolidinethiones, respectively. These results suggest that indole glucosinolates do not give rise to nitriles as readily as the other glucosinolates of rapeseed. Nevertheless, to avoid the possibility of nitrile production when analyzing rapeseed, McGregor (23) modified the method of Johnston and Jones (156), inactivating endogenous myrosinase and cofactors by heating and maximizing yields of thiocyanate ion by adding sodium hydroxide following myrosinase hydrolysis. Both brown mustard and rapeseed were observed to release thiocyanate ion, so it was recommended that measurement of isothiocyanates and oxazolidinethiones be complemented by measurement of thiocyanate ion to determine total glucosinolate content (23). This is particularly true of the new, low-glucosinolate varieties of rapeseed where thiocyanate was observed to constitute as much as 43% of the aglucone hydrolysis products (23).

Oxidation of thiocyanate ion has also been used to analyze for indole glucosinolate content. The method originally published by Aldridge (162, 163) involving reaction with bromine, pyridine, and benzidine as chromophore, was adopted by Michajlovskij and Langer (164) to analyze the thiocyanate content of brassicas. Josefsson (19) preferred this oxidation method over the ferric thiocyanate complex method of Johnston and Jones (156) because it gave higher results when applied to brassicas. Gierschner and Baumann (165) used barbituric acid, as the chromophore rather than benzidine, a potent

carcinogen. Jürges and Thies (166) modified this latter method for the routine determination of large numbers of samples of *Brassica* leaves and seed required by plant breeding programs. Thiocyanate ion was purified by binding it to a column of Sephadex QAE-A-25, then eluting with potassium chloride.

Raghavan and co-workers (167) used oxidation of thiocyanate to analyze for 4-hydroxybenzyl glucosinolate in yellow/white mustard. Benzidine was used as the chromophore. Sensitivity was reputed to be 10-fold greater than ferric ion colorimetric methods. Raghavan and co-workers (168) have also used iodometric analysis to determine the 4-hydroxybenzyl glucosinolate content of yellow/white mustard. Selectivity for 4-hydroxybenzyl glucosinolate was reputed to make the method useful for analysis of mixed mustard flours containing black mustard (allyl glucosinolate).

Recently, thiocyanate ion in animal tissues has been determined by oxidation followed by gas chromatography (169–171). Although this approach has yet to be applied to the determination of glucosinolate content, it appears to have potential for increasing sensitivity. Detection of 0.01 μg/g of thiocyanate ion has been claimed for biological fluids.

(c) *Oxazolidinethiones.*—Glucosinolates with a beta hydroxyl group in their side chain produce isothiocyanates which cyclize in polar solvents to form oxazolidinethiones. Oxazolidinethiones are not steam-volatile and thus can not be determined by methods such as the gravimetric, argentimetric or iodometric which involve steam distillation. Rapeseed contains both (R)-2-hydroxy-3-butenyl glucosinolate (progoitrin) and 2-hydroxy-4-pentenyl glucosinolate (gluconapoleiferin), while crambe contains epimeric (S)-2-hydroxy-3-butenyl (epiprogoitrin) glucosinolate (172, 173).

In 1949, Astwood and co-workers (174) reported that oxazolidinethiones absorb in the ultraviolet region and they developed a method for their measurement based on this absorption. Wetter (175), in analyzing for the glucosinolate content of rapeseed, supplemented his argentimetric method with a modification of Astwood's method to include oxazolidinethione-producing glucosinolates. Later, when Youngs and Wetter (44) introduced gas chromatography to facilitate determination of the glucosinolate content of rapeseed, they retained the use of ultraviolet absorption to measure oxazolidinethiones. When Wetter and Youngs (155) subsequently employed ultraviolet absorption of thioureas to determine the glucosinolate content of rapeseed, they took advantage of the similar absorption of thioureas and oxazolidinethiones to measure both simultaneously. An aliquot of methylene chloride containing isothiocyanates released by myrosinase was placed in ethanolic ammonia. While isothiocyanates of 3-butenyl and 4-pentenyl glucosinolates reacted with ammonia to form thioureas, isothiocyanates of 2-hydroxy-3-butenyl and 2-hydroxy-4-pentenyl glucosinolates cyclized in the polar solvent to form oxazolidinethiones. Oxazolidinethiones could be measured separately by placing the aliquot of methylene chloride in ethanol rather than ammoniacal solvent. Appelqvist and Josefsson (43) measured the thioureas and oxazolidinethiones separately by first selectively extracting the isothiocyanates with isooctane, then extracting the oxazolidinethiones with ether. Langer and Gschwendtova (143) extracted the isothiocyanates with ether, then divided the extract into 2 aliquots. Volatile isothiocyanates were evaporated from one of the aliquots to measure the residual oxazolidinethiones. The other aliquot was reacted with ammonia to obtain the combined thiourea and oxazolidinethione content. VanEtten and co-workers (176) have also determined 2-hydroxy-3-butenyl glucosinolate in crambe by extracting the oxazolidinethione into ether and measuring its ultraviolet absorbance.

Maheshwari and co-workers (90, 91) have used high performance liquid chromatography to separate the oxazolidinethiones in rapeseed meals. As little as 0.01 μg/g meal was detected by ultraviolet measurement.

(d) *Nitriles.*—Measurement of enzymatically released nitriles has not seen widespread use for determining glucosinolate content of agricultural crops or commodities. Daxenbichler and co-workers (29) developed a method for measuring the nitriles of crambe, based on infrared absorption. The same laboratory later developed a gas chromatographic method to measure the nitrile, epithionitriles, and isothiocyanate released by autolysis of 2- hydroxy-3-butenyl glucosinolate in crambe (177). Following hydrolysis with the endogenous enzyme and cofactors, the released products were extracted into methylene chloride containing methyl palmitate as internal standard, then separated by temperature programmed chromatography on columns of either 3% Apiezon L or 1% EGSS-X. EGSS-X separated the 2 diastereomeric forms of the episulfide, 1-cyano-2-hydroxy-3,4-epithiobutane. The method was useful for determining the fate

of 2-hydroxy-3-butenyl glucosinolate during processing of foods and feed because it measures all of the myrosinase hydrolysis products. It was later modified to measure μg/g levels of these products in body fluids (178) and the nature and content of other organic nitriles found in cabbage (89) and processed cabbage (179).

Brak and Henkel (180) tried to quantitatively convert glucosinolates to nitriles by using acidic hydrolysis. Thies (49) has proposed a method for determining the glucosinolate content of rapeseed using dry heating (pyrolysis) rather than enzymatic hydrolysis to produce nitriles. The analysis was facilitated by forming the nitriles inside an aluminum capsule in the injection port of a gas chromatograph and by using a nitrogen specific detector.

(e) *Sulfate.*—Insofar as is known, all glucosinolates give rise to equimolar amounts of sulfate, independent of the conditions of myrosinase hydrolysis. Total glucosinolate content of agricultural crops or commodities can thus be indirectly determined by measurement of the sulfate released under uncontrolled hydrolysis conditions. Both gravimetric (181–183) and titrimetric (176, 184, 185) measurements have been used. However, in general, methods involving measurement of sulfate are time consuming and tedious. Lack of precision and relatively large sample size requirements have prevented their widespread use.

Realization that 4-hydroxybenzyl glucosinolate of yellow/white mustard does not give rise to a steam-volatile isothiocyanate led Bauer and Holle (181) to develop a method for the gravimetric measurement of the sulfate. The glucosinolate was hydrolyzed with myrosinase and, following hydrolysis, the solution was clarified by the addition of potassium ferrocyanide, zinc acetate in acetic acid, and filtration. Sulfate was precipitated as barium sulfate by the addition of barium chloride, and weighed. Background level of free sulfate was determined by first inactivating endogenous myrosinase with boiling water. McGhee and co-workers (182) applied the method to crambe; Josefsson and Appelqvist (183) analyzed rapeseed, but without the clarification step.

Terry and Curran (184) used titration of myrosinase-released sulfate to determine the glucosinolate content of both yellow/white and brown mustard. Following myrosinase hydrolysis, the hydrolysate was freed of phosphate by the addition of magnesium carbonate and ammonia and filtering. Sulfate was precipitated with benzidine, and the benzidine sulfate was dissolved in a measured volume of sodium hydroxide and back-titrated with sulfuric acid to a methyl red end point.

VanEtten and co-workers (176) have used a modification of the method of Fritz and Yamamura (185) to determine the glucosinolate content of crambe. The hydrolysate was purified by ion exchange on IR 120-H, the eluate was diluted with ethanol, and the sulfate was titrated with barium perchlorate, using thorin as indicator. McGregor (23) modified the method to apply to small samples of rapeseed by measuring the end point with a spectrophotometer. Croft (186) recently examined titration of sulfate as a simple, routine method for determining total glucosinolate content of rapeseed. Glucosinolates were first aqueous-extracted and the extract was clarified as described by Bauer and Holle (181). The extract was then adjusted to pH 6 with alkali, myrosinase was added and, after hydrolysis, the sulfate was titrated to pH 6.

(f) *Glucose.*—Measuring glucose to determine the total glucosinolate content of agricultural crops or commodities is based on the fact that, with only one exception (5), glucose has been found as the sugar moiety of all known glucosinolates. Schultz and Gmelin (187) cleaved the glucosinolates with strong sulfuric acid, then measured the glucose released colorimetrically with anthrone reagent. However, most methods measure glucose after enzymatic rather than chemical breakdown of the glucosinolates. Two enzyme systems have been used to measure glucose, one employing hexokinase and glucose-6-phosphate dehydrogenase (188, 189), the other glucose oxidase and peroxidase (189–192).

The hexokinase/glucose-6-phosphate dehydrogenase system was originally applied to determine the glucosinolate content of rapeseed by Lein and Schon (188, 189). Following myrosinase-hydrolysis of seed meals, glucose was reacted with adenosine triphosphate under the action of hexokinase to form glucose-6-phosphate which then, under the action of glucose-6-phosphate dehydrogenase, reduced nicotinamide adenine dinucleotide phosphate. The decrease in ultraviolet absorption resulting from the reduction was measured. Background level of free glucose was determined using an unhydrolyzed sample. The method was reported to be accurate to within 1% glucose and sufficiently sensitive to allow determination of the glucosinolate content of one rapeseed cotyledon, an asset to plant breeding for reduced glucosinolate content.

For less precise, less sensitive, but more efficient analysis of rapeseed, Lein (189) used a rapid, simple, and specific test paper (Tes-Tape) developed by Comer (193) for semiquantitative glucose determination. The test paper uses the glucose oxidase/peroxidase system. Glucose reacts with the glucose oxidase-impregnated paper to produce gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with peroxidase to turn the chromogen *o*-tolidine from colorless to blue. Yellow dye in the paper results in the paper turning from yellow to green. The test paper has been extensively used to screen large numbers of seed samples in plant breeding programs aimed at developing low-glucosinolate varieties of rapeseed. It has been effective because mature seed has a low content of free glucose. Higher levels of free glucose prevents application of the test paper to vegetation.

A limitation of the glucose oxidase/peroxidase system when applied to crude plant extracts has been the apparent inhibition of the peroxidase enzyme by phenolics present in the extracts (188). Bjørkman (190) avoided the problem by purifying glucosinolate extracts using ion exchange. VanEtten and co-workers (191) treated extracts with charcoal to adsorb the inhibitor before colorimetric analysis. This method was subsequently automated to facilitate analysis of large numbers of analyses associated with rapeseed breeding programs (194). The problem of inhibition was avoided to some extent by VanEtten and co-workers (191) when using the glucose-specific test paper by allowing the extract to migrate along the paper to separate the inhibitor from glucose. However, McGregor and Downey (192) found that addition of charcoal could enhance the sensitivity and reproducibility of the test paper facilitating the identification of low glucosinolate rapeseed. Seed was crushed in a small mortar, water was added, and glucose was released by autolysis. Powdered charcoal was then added to adsorb the inhibitor and, after dilution with water, the solution was filtered by addition of a filter paper disc to the surface of the mixture. A strip of test paper was laid on the filter paper and the color development was assessed after 3 min.

MacGibbon and co-workers (195) have screened *Brassica* seed extracts for glucosinolate content by paper chromatography of the glucose, development of the chromatograms by spraying with silver nitrate and sodium hydroxide, and quantitation by comparison of the size and intensity of the spots with those of known glucose content. Although reputed to be several times more sensitive than the glucose oxidase/peroxidase test paper method, comparison was made without using charcoal to enhance the sensitivity of the glucose oxidase/peroxidase test paper.

Olsson and co-workers (196) developed a method for measuring glucose which they considered particularly suited to the determination of the glucosinolate content of low glucosinolate rapeseed, rapeseed protein concentrates, and derived foods and feeds. Enzymatically released glucose was quantitated by gas chromatography of the trimethylsilyl derivative with myo-inositol as internal standard. Although considerably more sensitive than either the hexokinase/glucose-6-phosphate dehydrogenase or the glucose oxidase/peroxidase systems, the full potential cannot be reached when applied to *Brassica* seed extracts without making time-consuming corrections for background levels of free glucose and a compound with the same retention time as myo-inositol (McGregor, unpublished).

VanEtten and co-workers (178) have removed background amounts of free glucose from extracts by binding the glucosinolates onto an ion exchange column, washing through the glucose and other contaminants, and then hydrolyzing by adding myrosinase directly to the column. An asset of this approach is the option of extracting the aglucones with methylene chloride for gas chromatography to provide compositional data. Heaney and Fenwick (197) modified the method for routine determination of total glucosinolate content of either seed or vegetative material. Analysis was facilitated by improving flow control of the columns, eliminating the need for constant monitoring, and reducing sample size, analysis time, and cost.

Glucosinolates

In recent years, there has been a significant advance in methodology for determining the glucosinolate content of agricultural crops or commodities without involving myrosinase hydrolysis. Two approaches exploit the potential of improved instrumentation, notably gas chromatography and high performance liquid chromatography, for greater resolution and sensitive quantitation to determine both glucosinolate composition and content. A third uses colorimetric analysis to determine total glucosinolate content.

(a) *Gas Chromatography.*—Gas chromatography of trimethylsilylated desulfoglucosinolates, as previously described, has not only been used to determine glucosinolate composition, but has

also been applied to the determination of glucosinolate content. To quantitate results, Underhill and Kirkland (48) made the assumptions that all glucosinolates were derivatized to the same extent, and that mole composition was proportional to the peak areas divided by the number of carbon atoms in the respective trimethylsilyl derivative. Heaney and Fenwick (109) have subsequently found that while this appeared to apply for many glucosinolates, 1-methoxy-3-indolyl glucosinolate had a considerably lower response, almost half that of 3-indolylmethyl glucosinolate. Although the reason for this is not fully understood, recent investigation (111) using combined gas chromatography–chemical ionization mass spectrometry has demonstrated the importance of derivatization conditions. At high temperatures (>140°C), 1-methoxy-3-indolylmethyl glucosinolate was considerably degraded, while at lower temperatures (<100°C), 3-indolylmethyl glucosinolate was incompletely derivatized. It was suggested that derivatization for 20 min at 120°C was a reasonable compromise for quantitation of these 2 glucosinolates. Thies (unpublished) has recently employed 1-methyl imidazole rather than pyridine for derivatization of these and other glucosinolates.

Quantitative analysis of trimethylsilylated desulfoglucosinolates can be influenced by a memory effect which occurs when samples with dissimilar composition or content are analyzed consecutively, or when the chromatograph has not been used for a period of time (104). In such cases, samples have to be chromatographed more than once to obtain reproducible detector response.

Gas chromatography of trimethylsilylated desulfoglucosinolates on a packed column of OV-7 has been used to separate and quantitate most of the glucosinolates found in *Brassica* species including whose which, on myrosinase hydrolysis, give rise to isothiocyanates, oxazolidinethiones, and thiocyanate ion (41, 109). Notable exceptions are 3-methylsulfinylpropyl glucosinolate, which forms multiple peaks when derivatized (109), and 3-indolylmethyl and 4-hydroxy-3-indolylmethyl glucosinolates which have recently been reported not to be separated on a packed column of OV-7 but which may be separated on a capillary column of SE-54 (123).

(b) *High Performance Liquid Chromatography.*—High performance liquid chromatography affords the advantage of direct determination of glucosinolates not only for glucosinolate identification but also for quantitation. Although not yet fully applicable to quantitative analysis the reverse phase ion-pairing method described for separation of glucosinolates (122) and the reverse-phase method described for desulfoglucosinolates (113) indicate the potential. Particular advantages are the avoidance of acidic and basic conditions, and high temperatures, which can cause glucosinolate decomposition (63, 198). Judicious selection of appropriate pH, the nature and concentration of the counter ion, the counter ion concentration, or the composition of the mobile phase should resolve the current problems of incomplete resolution.

(c) *Colorimetry.*—Recently, Thies (199) has developed a method whereby the glucosinolate content of rapeseed can be estimated by reaction with sodium tetrachloropalladate to form a colored complex. Only a few seeds are required. Glucosinolate content is estimated either with a colorimeter or visually by comparison with seed samples of known glucosinolate content. The method is simple and rapid and apparently more sensitive than the glucose oxidase/hexokinase glucose-specific test paper method of Lein (189). It is potentially of value for rapeseed breeding programs aimed at virtually eliminating glucosinolates from the seed.

Standardization of Methodology and Reporting of Results

An unfortunate consequence of the diversification of analytical methods for determining glucosinolate content has been the wide variety of means used to report results. This often makes comparison of results difficult and sometimes impossible. For example, McGregor (200), in carrying out an interlaboratory survey on the determination of the glucosinolate content of rapeseed meal, found that some laboratories reported results as a weight or weight percent of individual isothiocyanates, while others reported results as equivalents of either 3-butenyl or allyl isothiocyanate. Still others reported results in micromoles. Some preferred to report results on an oil-extracted moisture-free basis, others on an oil-extracted air-dry (7% moisture) basis. Often different ways of reporting results are prescribed by the different methods in use in the various laboratories.

To standardize the means of reporting results in the rapeseed industry, participants at an international symposium on the analytical chemistry of rapeseed and its products (201), recommended that where possible results should be reported in micromoles per gram of oil-extracted air-dry (7% moisture) basis. Reporting results on

"An unfortunate consequence of the diversification of analytical methods for determining glucosinolate content has been the wide variety of means used to report results. This often makes comparison of results difficult and sometimes impossible. A current problem with attempts to standardize quantitative glucosinolate determination is the lack of pure glucosinolates with which to establish accuracy."

an air-dry basis was recommended to facilitate regulatory and quality control work by eliminating the need to determine dry matter of seed meal samples. Whether for seed meal samples or vegetative samples, reporting of results in micromoles would greatly assist comparison and interpretation of results.

To further facilitate regulatory and quality control within the Canadian rapeseed industry, an approved method of analysis has recently been adopted by Canadian Grain Commission (202). It is essentially the method of Thies as modified by Heaney and Fenwick (109). Benzyl glucosinolate is recommended for use as internal standard so that admixtures of rapeseed and brown mustard or stinkweed, which contain allyl glucosinolate, or yellow/white mustard or wild mustard, which contain 4-hydroxybenzyl glucosinolate, can be detected.

A current problem with attempts to standardize quantitative glucosinolate determination is the lack of pure glucosinolates with which to establish accuracy. Few glucosinolates have been synthesized or isolated, and those that have been available have differed in purity depending on commercial source or storage time (108). At present, sinigrin is commercially available from Aldrich Chemical Co. (USA) and Apin Chemicals (UK), while a number of glucosinolates are included in the Roth Co. (GFR) catalog. Hanley, Heaney, and Fenwick (manuscript submitted) have recently developed methods for the isolation of larger amounts of glucosinolates from plant sources. Benzyl glucosinolate has been isolated from papaya seed (*Carica papaya* L.), although *Tropeolum majus* L. seed is probably a better and cheaper source (McGregor, unpublished observation). Benzyl glucosinolate has also been synthesized (4, 204), but due to low yields it is an expensive means of procurement (McGregor, unpublished observation).

Thies (108) has recently suggested that desulfoglucosinolates may be more useful for both identification and calibration purposes because they can be more easily obtained in pure form than their parent glucosinolates and are more stable. Minchinton and co-workers (113) have subsequently described a high performance liquid chromatography method which they claim is suitable for isolating milligram amounts of a number of desulfoglucosinolates with purity suitable for use as primary standards.

Overview and Future Outlook

Analytical methodology for glucosinolates has undergone a considerable evolution, particularly over the past 3 decades. As a result a vast array of methods have been developed. While many of the older methods are still useful for complimenting newer methods of glucosinolate identification, or for determining glucosinolate content in specific research, regulatory, or quality control applications, some of the newer methods stand out as being particularly suited to glucosinolate identification or quantitation.

Fast atom bombardment mass spectrometry (121) seems particularly suited to aid glucosinolate identification. Relatively little fragmentation coupled with the unique molecular weights of the vast majority of individual glucosinolates facilitates identification of molecular ions in the positive ion mode. Mixtures of glucosinolates in crude plant extracts can be identified with negative ion fast atom bombardment (117, 118).

For simple and rapid determination of total glucosinolate content, colorimetric measurement of glucose released by myrosinase hydrolysis seems most appropriate. The glucose-specific test paper method (189) has been the method of choice by plant breeders for screening large numbers of samples to develop low-glucosino-

late rapeseed varieties. Modified with the addition of carbon (192), it has been sufficiently sensitive and reproducible to identify low-glucosinolate rapeseed in commerce. When combined with ion exchange purification (60, 197), it is suitable for routine determination of the glucosinolate content of either seed or vegetative material.

For more precise and discriminating analysis of glucosinolate content, temperature programmed gas chromatography of trimethylsilyl derivatives (109) appears to be most appropriate. In addition to glucosinolates which give rise to isothiocyanates and oxazolidinethiones, glucosinolates which yield thiocyanate ion can be detected and quantitated. However, this method can not be applied to 3-methylsulfinylpropyl glucosinolate. For *Brassica* leaves and other tissues in which this glucosinolate is present, gas chromatography of isothiocyanates produced under controlled myrosinase hydrolysis (53) is better suited. The ability of this method to detect oxazolidinethiones, nitriles, and epithionitriles in addition to isothiocyanates also makes this method applicable to foods or feeds where hydrolytic conditions have not been controlled.

Analytical methodology for glucosinolates is continuing to evolve and improve. Undoubtedly new methods will appear which will further facilitate analysis. For rapid and simple measurement of total glucosinolate content, the use of palladium (199) offers promise of increased sensitivity and precision. For qualitative and quantitative analysis of complex glucosinolate mixtures, high performance liquid chromatography (113, 122), gas chromatography of trimethylsilyl derivatives (108, 109), or a combination of both (63, 198) likely has further potential. These approaches are consistent with a recent trend away from the production and measurement of enzymatic or chemical breakdown products and favor measurement of the glucosinolates themselves, or derivatives thereof.

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PLANTS

Electrothermal Atomic Absorption Spectroscopic Determination of Chromium in Plant Tissues

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This method involves sample digestion in nitric, perchloric, and sulfuric acids. Chromium is concentrated by coprecipitation with ferric hydroxide. Redissolved iron is removed by liquid–liquid extraction, remaining silica is dissolved with hydrofluoric acid, and chromium is determined by electrothermal atomic absorption spectrometry. The sensitivity and detection limits for chromium approach those given by the manufacturers of the various instruments. Recovery studies and analysis of standard materials show that this method is reliable.

There is no official AOAC method for the determination of chromium in plants. The need for such a method became evident when 2 interlaboratory studies on determination of chromium in brewer's yeast showed that a number of methods were inaccurate (1). Thus the possibility exists that much of the work related to the essential function of chromium in animal nutrition is erroneous. Three major problems leading to incorrect results are contamination by laboratory dust during analysis (2), adsorption of chromium by silica (3–7) and graphite (8), and background correction problems associated with electrothermal atomization of chromium from complex matrices (9, 10). Low recoveries of chromium have also been attributed to the formation and volatilization of chromyl chloride from nitric–perchloric acid digestion mixtures. This compound can be formed in a number of ways (11), but conditions in a nitric–perchloric digestion do not appear to meet the necessary requirements.

The present method is based on wet digestion of the sample followed by isolation and concentration of chromium(III) by coprecipitation with iron (12, 13). The iron is then extracted into 4-methyl-2-pentanone from an 80% hydrochloric acid solution and discarded. Chromium(III) remains in the aqueous phase. Silica, which also remains in the aqueous phase, is then dissolved with hydrofluoric acid. Chromium is determined by electrothermal atomic absorption spectrometry.

METHOD

Apparatus

(a) *Atomic absorption spectrometer.*—With graphite furnace accessory (Perkin-Elmer Model 603 with HGA 2100 furnace, or equivalent).

(b) *Digestion tubes.*—Approximately 20 × 175 mm.

(c) *Polyethylene centrifuge tubes.*—Calibrated to 15 mL.

(d) *Polyethylene pipets.*—Adjustable to 5 mL.

Reagents

All reagents are reagent grade unless otherwise stated. Use distilled or deionized water.

(a) *Sulfuric acid.*—Ultrex or equivalent.

(b) *Nitric acid.*—Redistilled in glass. Distill ca 90% of total.

(c) *Perchloric acid.*—70%, double distilled from Vicor (G. Frederic Smith Chemical Co., No. 230, or equivalent).

(d) *Hydrochloric acid.*—Dilute as needed.

(e) *Hydrofluoric acid.*—48%.

(f) *Ammonium hydroxide.*—Dilute as needed.

(g) *Sodium sulfite.*—1% (w/v). Prepare fresh in water each day.

(h) *4-Methyl-2-pentanone (MIBK).*—Eastman No. 416, or equivalent.

(i) *Methyl red.*—0.02 g in 60 mL ethanol. Dilute to 100 mL with water.

(j) *Chromium standard solution.*—Dissolve 0.3736 g K_2CrO_4 in water and dilute to 1 L. Dilute this solution to a working concentration of 0.1 μg Cr/mL. Commercial atomic absorption standards are also satisfactory solutions from which to make appropriate dilutions.

(k) *Iron solution.*—Dissolve 10 g $FeCl_3 \cdot 6H_2O$ in 10 mL concentrated HCl. Add 0.5 mL 1% Na_2SO_3 and mix. Extract 3 times with 20 mL MIBK, combining organic phases after each extraction. Wash organic phase with 3 mL 6N HCl

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and extract twice with 50 mL portions of water. Save aqueous phase and dilute to 500 mL with water.

Glassware

Clean digestion tubes and glass boiling beads by heating 15 min beyond fuming with H_2SO_4 and $HClO_4$ (100 + 1), the first time they are used. Soak polyethylene materials for 24 h in 10% HNO_3–H_2SO_4. Rinse everything with 10% HCl followed by water. Use for chromium determinations only.

Preparation of Sample

Dry samples at 70°C to constant weight. Do not grind samples unless essential for subsampling procedures. If grinding is necessary, a Wiley mill, equipped with chromium-plated 20 mesh screen, is recommended (3). The chromium-plated screen appears to have a harder surface than the stainless steel screen. Use caution; both materials can be major sources of contamination. Other methods of grinding have not been investigated.

Preparation of Standard Curve

Prepare chromium standards to bracket chromium concentrations in samples. Carry 2 reagent blanks through method. Standards do not need to be digested but should contain 1 mL $HClO_4$ and 0.5 mL H_2SO_4.

Determination

Weigh ≤1 g (dry weight) sample into digestion tube. Add 2 glass beads, 0.5 mL H_2SO_4, 5 mL HNO_3 per 0.5 g sample, and 1 mL $HClO_4$. Digestion will be easier if mixture is left at room temperature overnight. Heat slowly until all easily oxidized material is in solution; then increase heat. Continue digestion at least 10 min beyond appearance of $HClO_4$ fumes. If digest darkens just before appearance of $HClO_4$ fumes, immediately add 0.25 mL HNO_3 and continue heating. Repeat if necessary. If extra HNO_3 is used, include appropriate reagent blank. Let digest cool. Clean rim and outside top of tube. Transfer digest, including particulate matter, to 15 mL polyethylene centrifuge tube with four 1 mL water rinses. Add 0.05 mL 1% Na_2SO_3 and mix. Add 1 mL iron solution and mix. Add 2 mL NH_4OH and mix. This should increase pH above 8. Age precipitate at least 10 min. Centrifuge and decant carefully. Resuspend precipitate in ca 6 mL water. Centrifuge and decant. Add 1 mL 80% HCl and 1 drop of methyl red (for a phase marker) and let precipitate dissolve.

Table 1. Recovery of inorganic chromium digested in HNO_3–$HClO_4$ and HNO_3–$HClO_4$–H_2SO_4 systems [a]

Acids	Chromium retained at 200°C, % [b] 5 min	1 h
$HNO_3 + HClO_4$	99.5 ± 0.5	99.7 ± 0.5
$HNO_3 + HClO_4 + H_2SO_4$	100 ± 0.5	99.5 ± 0.5

[a] Nitric acid was distilled from 20 × 175 cm tubes and temperature was adjusted so that $HClO_4$ or $HClO_4$–H_2SO_4 just continued to boil.
[b] ±1 SD.

Extract twice, first with 5 mL and then with 3 mL portions of MIBK. Discard MIBK (top) phase which contains iron. (Use clear polyethylene-tip pipet to make phase separation in this extraction.) Add 1 mL $CHCl_3$ and mix; then centrifuge. If layer of solids is seen at $CHCl_3$–water interface, add 0.1 mL HF. Do not shake. Let this material dissolve. Dilute aqueous phase to volume (i.e., 8 mL), mix, and centrifuge. Determine chromium in aqueous phase, using electrothermal atomic absorption spectrometry (HGA). Use the following settings on the Perkin-Elmer Model 603 atomic absorption spectrophotometer: wavelength, 357.9 nm; HGA 2100 drying, 110°C for 20 s; charring, 1000°C for 10 s; atomizing, 2700°C for 9 s; argon flow, normal; flow rate, 40 through Brooks tube size R-2-15A (CO). No background correction is necessary. Use pyrolized tubes.

Results and Recommendations

Chao and Pickett (4) studied the volatilization of Cr from various acid mixtures and found that Cr is volatilized from HNO_3 or HNO_3–$HClO_4$ acid mixtures but not from a H_2SO_4–$HClO_4$ acid mixture when heated 3 h at 200°C. They also reported that no Cr is lost from a HNO_3–$HClO_4$ acid mixture if the digestion is carried out in a volumetric flask. The use of 20 × 175 mm culture tubes also eliminates the loss of Cr from HNO_3–$HClO_4$ digestions, as shown in Table 1. However, to eliminate any possibility of vola-

Table 2. Recovery of inorganic chromium added to digested rye grass

Sample	Absorbance [a]
0.1 μg Cr	0.138 ± 0.007
0.48 g rye grass	0.228 ± 0.005
0.48 g rye grass + 0.1 μg Cr	0.367 ± 0.008

[a] ±1 SD.

Table 3. Concentration of chromium in NBS biological standards

Sample	SRM No.	ppm Cr[a]	
		NBS value	This method
Wheat Flour	1567	—	0.24 ± 0.01 (3)[b]
Brewer's Yeast	1569	2.1 ± 0.05	2.02 ± 0.10 (22)
Spinach	1570	4.6 ± 0.3	4.47 ± 0.40 (10)
Orchard Leaf	1571	2.6 ± 0.3	2.56 ± 0.11 (19)
Tomato Leaf	1575	4.5 ± 0.5	4.48 ± 0.19 (13)

[a] ±1 SD.
[b] Number of values.

tilization, a small amount of H_2SO_4 is added to the digestion mixture (4).

Standards covering the range of 0.05 to 1 μg Cr and a blank were carried through the procedure. The final dilution was 8 mL. Ten μL samples were injected into the pyrolized graphite tube and triplicate readings were obtained for an average value. After the absorbance value for the blank was subtracted the equation describing the standard curve was absorbance = 0.436 × μg Cr − 0.002.

A 4 g sample of rye grass, containing more than 0.1% Si, was digested in the presence of 1 μg added Cr. This was used as a plant-derived matrix high in Si that could be uniformly subsampled for a Cr recovery study. The digest was diluted to 25 mL and six 3 mL subsamples were taken for chromium analysis. Three were analyzed as taken, and 3 others received 0.1 μg Cr each before analysis. Table 2 shows that replication of results and recovery of the added Cr were excellent.

Analysis of five NBS biological standards shows that both accuracy and precision of this method are very good (Table 3). Data were collected by 2 analysts over a period of several months.

The advantages of this procedure are as follows: Only standard laboratory equipment is needed; the sample is in contact with only 2 containers during the procedure; both containers have small openings to help prevent airborne contamination; and a relatively simple matrix is presented to the graphite tube so background correction is unnecessary and a lower detection limit is possible.

It is emphasized that extreme caution should be used when working with perchloric and hydrofluoric acids. The former is a strong oxidizing agent and the latter is extremely corrosive.

It is recommended that this method be subjected to a collaborative study.

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DECOMPOSITION IN FOODS

High Pressure Liquid Chromatographic Determination of Putrefactive Amines in Foods

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A high pressure liquid chromatographic (HPLC) procedure is described for determining the following putrefactive amines: histamine, tyramine, putrescine, cadaverine, tryptamine, and β-phenylethylamine. The amines were extracted from tuna or cheese with methanol. Futher cleanup was performed by sequential extractions with butanol and HCl. The acid extract was dried, and residues were derivatized with dansyl chloride. HPLC separations were performed on an Ultrasphere-ODS column at 33°C. A gradient elution program was used; the total elution time was less than 17 min. Linear standard curves with high correlation coefficients were obtained. The procedure allowed good recoveries of histamine, tyramine, putrescine, and cadaverine; recoveries of tryptamine and β-phenylethylamine were lower but constant. With this method, some swiss cheese samples were found to contain considerable amounts of histamine, tyramine, putrescine, cadaverine, and β-phenylethylamine. Canned tuna samples had very low levels of these amines. Since the presence of amines at high levels has been associated with tuna decomposition, this method may be useful in identifying decomposed fish.

Food poisoning episodes associated with the consumption of products containing high levels of histamine have been reported. Most often, scombroid fish (1–3) and cheese (4, 5) are implicated in the outbreaks. The high levels of histamine are formed by microbial decarboxylation of histidine. A relationship between histamine levels and fish spoilage was first observed by Geiger (6, 7). In a study by Meitz and Karmas (8), 5 amines (histamine, putrescine, cadaverine, spermine, and spermidine) were analyzed by high pressure liquid chromatography (HPLC) to generate a chemical index of tuna decomposition. Staruszkiewicz and Bond (9) developed a gas-liquid chromatographic procedure to study the content of putrescine and cadaverine in decomposed fishery products and cheese products. Amine levels were shown to be a good index of fish decomposition.

Evidence for the involvement of histamine in scombroid fish poisoning outbreaks is circumstantial. Symptoms of scombroid fish poisoning include facial flushing, throbbing headache, sharp or peppery taste, burning sensation, cardiac palpitation, dizziness, urticaria, and gastrointestinal distress (10). Most of these symptoms resemble those produced by intravenous histamine administration, and antihistamine therapy has been useful in these cases. Oral administration of large doses of histamine caused some of the same characteristic symptoms of scombroid poisoning (11) but with markedly less severity (10). This relative lack of oral histamine toxicity is possibly due to the presence of histamine-metabolizing enzymes in the intestines. Two primary enzymes for histamine metabolism are histamine-*N*-methyltransferase (HMT) and diamine oxidase (DAO) (12). The difference in response to histamine in spoiled tuna and oral administration of pure histamine still remains unexplained. One possible explanation would be the chemical potentiation of histamine toxicity by the coincident existence of inhibitors of the intestinal histamine-metabolizing enzymes in cheese and spoiled tuna. In a study by Taylor and Lieber (13), many inhibitors of HMT and DAO were identified. Some of these identified inhibitory chemicals might be found in tuna along with histamine. Among the most potent inhibitors of HMT and DAO were a number of amines including tyramine, β-phenylethylamine, tryptamine, cadaverine, and putrescine, which are decarboxylation products of tyrosine, phenylalanine, tryptophan, lysine, and ornithine, respectively.

To detect and identify foodborne inhibitors of histamine-metabolizing enzymes, a reliable procedure is needed. However, no such method existed for the simultaneous detection of histamine, tyramine, β-phenylethylamine, tryptamine, putrescine, and cadaverine in foods or biological samples. In this study, a HPLC method was developed for qualitative and quantitative analysis of these amines.

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Experimental

Apparatus

(a) *Liquid chromatograph.*—Consists of 2 Model 100A solvent metering systems, Model 420 microprocessor, Model 500 automatic sampler, and Model 210 sample injection valve (Beckman Instruments, Inc., Berkeley, CA 94710).

(b) *UV detector.*—Model 440 (Waters Associates, Milford, MA 01757).

(c) *Data processor.*—Model C-RIA (Shimadzu Corp., Kyoto, Japan).

(d) *HPLC column.*—Ultrasphere-ODS, 25 cm × 4.6 mm id, 5 μm particles (Altex Scientific, Inc., Berkeley, CA 94710).

(e) *Water jacket for HPLC column.*—1/4 in. od, 250 mm long (Altex Scientific).

(f) *Filtration systems.*—All-glass filter holder for solvent filtration, 47 mm, includes 300 mL sample reservoir and 1 L receiving flask; centrifugal filter tubes, 12 cm long, 1.2 cm diameter; Nylon-66 membrane filter disk, 0.45 μm pore size, 8.5 mm and 47 mm diameters (Rainin Instrument Co., Inc., Woburn, MA 01801).

Reagents

(a) *Mobile phase.*—Solvent A: methanol and acetonitrile in equal proportion (distilled in glass, Burdick & Jackson Laboratories, Muskegon, MI 49442). Solvent B: 0.33mM phosphoric acid in deionized distilled water. Filter all solvents with Nylon-66 membrane filter discs (0.45 μm pore size, 47 mm diameter) before use.

(b) *Standard solution.*—Dissolve 165.7 mg histamine dihydrochloride, 126.7 mg tyramine hydrochloride, 122.8 mg tryptamine hydrochloride, 130.1 mg β-phenylethylamine hydrochloride, 182.9 mg putrescine dihydrochloride, and 171.4 mg cadaverine dihydrochloride (Sigma Chemical Co., St. Louis, MO 63178) in deionized distilled water and dilute to 10 mL. This results in a standard solution with 10 mg free base form of each amine per mL.

(c) *Internal standard solution.*—Stock solution: 1 mg/mL. Dissolve 10 mg 1,7-diaminoheptane (Aldrich Chemical Co., Milwaukee, WI 53233) in 10 mL deionized distilled water.

(d) *Dansyl chloride solution.*—Stock solution: 5 mg/mL. Dissolve 50 mg dansyl chloride (Sigma Chemical Co.) in 10 mL acetone.

Samples

Canned tuna samples packed in oil or water, and some of the swiss cheese samples were purchased from local supermarkets. Selected swiss cheese samples (19-1, 19-2, and 19-3) were obtained from the Food and Drug Administration, Dairy and Fishery Technology Branch, Division of Food Technology.

Sample Preparation

(a) *Extraction of amines.*—This procedure is basically a combination with minor modifications of the official AOAC method, **18.066** (14), and Taylor et al. method (15). Homogenize 10 g well mixed food sample (tuna or cheese) with 50 mL methanol for 2 min. Transfer to 125 mL flask, rinse blender jar and lid with 10 mL methanol, and add rinsings to flask. Heat in water bath to 60°C and let stand 15 min at this temperature. Cool to room temperature and filter homogenate into 100 mL volumetric flask, using Whatman No. 1 paper. Wash precipitate on filter paper with more methanol until final volume reaches 100 mL. Dilute 1:10 with deionized distilled water. Transfer 5 mL diluted extract to test tube containing 1 mL 5N NaOH, and saturate extract with granular anhydrous Na_2CO_3. Extract amines with 6 mL water-saturated *n*-butanol, and mix vigorously. Transfer 3 mL butanol layer to test tube containing 3 mL 0.1N HCl, mix, and aspirate upper organic layer. Pipet 2 mL acid phase into test tube and evaporate to dryness under vacuum.

(b) *Derivatization of amines.*—To test tube with dried amines, add 0.5 mL saturated sodium bicarbonate, 1 mL dansyl chloride solution, and 10 μL internal standard solution. Cap and mix sample at least 15 s on Vortex mixer. Let mixture stand 1 h at 37°C or overnight at room temperature. Add 1 mL deionized distilled water to mixture and evaporate acetone under vacuum. Extract dansylated amines with three 3 mL portions of ethyl ether. Combine ether extracts and take to dryness under vacuum. Dissolve residues in 2 mL acetonitrile and filter sample using centrifugal filter tubes with Nylon-66 membrane filter disk. Inject 20 μL aliquot for HPLC analysis.

Chromatographic Separation

Use linear solvent program (gradient elution) from 60% solvent A to 80% solvent A in 16 min. Program flow rate from 1.5 mL/min to 2.5 mL/min in 16 min. Detect dansylated amines by setting wavelength of UV detector at 254 nm. Keep temperature of column constant at 33°C with water jacket.

Preparation of Standard Curves

Prepare standard curve for each amine by adding to 10 g good quality tuna various volumes

(0–1 mL in 0.1 mL increments) of standard amine solution. Tuna was considered good quality when none of the 6 amines was found by HPLC analysis. This results in tuna samples containing 0–100 mg/100 g of each amine. Carry out extraction and derivatization procedures as described above. Inject triplicate aliquots of each standard concentration and determine reproducibility of the method. Obtain plot of photometric response (relative peak area) vs concentration for each amine.

Recovery of Amines

To determine efficiency of methanol extraction, add 0.5 mL standard amine solution to 10 g tuna samples from each of 9 different cans of tuna and 0.5 mL standard amine solution to methanol extracts from each of these 9 cans. Carry out remainder of procedure as described above. Calculate percent recovery by comparing photometric responses of samples with amines added to tuna to samples with amines added to methanol extract. Do a blank determination of amines concentration on each of these 9 samples.

Prepare external standard by carrying different amounts of amines through dansylation procedure. Calculate efficiency of butanol–HCl extraction by comparing photometric responses of external standards to samples with amines added to methanol extract. Take these extraction efficiencies into account when analyzing food samples for unknown concentrations of amines.

Results and Discussion

A chromatogram of the mixture of amines is shown (Figure 1). The total elution time was less than 17 min. The amino acids found in tuna, which were not resolved, were the first 2 peaks on the chromatogram. The identities of these peaks were confirmed by analyzing various amino acids, i.e., histidine and lysine. 5-Dimethylaminonaphthalene-1-sulfonic acid, which was a hydrolysis product of dansyl chloride, was eluted at 6.43 min. This was followed by tryptamine (8.83 min), β-phenylethylamine (9.76 min), putrescine (12.43 min), cadaverine (13.13 min), histamine (14.33 min), 1,7-diaminoheptane (15.36 min), and tyramine (16.96 min). The amines were well separated with this gradient elution program. Isocratic elution was also investigated, with a mixture of 43% acetonitrile, 30% methanol, 26% water and 1% acetic acid; good separation was obtained. However, total elution time needed in such a system was 28 min,

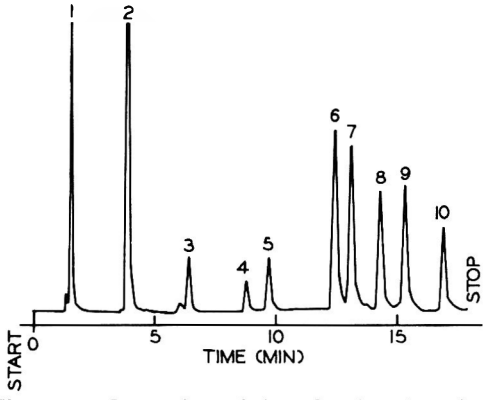

Figure 1. Separation of dansylated amines from spiked tuna by HPLC. 1 and 2, amino acids (unresolved); 3, 5-dimethylaminonaphthalene-1-sulfonic acid; 4, tryptamine; 5, β-phenylethylamine; 6, putrescine; 7, cadaverine; 8, histamine; 9, 1,7-diaminoheptane (internal standard); 10, tyramine.

and this longer analysis time resulted in some disadvantages such as broadening of peaks and solvent wastage.

The data for the standard curves were submitted to linear regression analysis; correlation coefficients and linear regression coefficients are shown (Table 1). A correlation coefficient greater than 0.99 was obtained with every curve except tyramine, which had a correlation coefficient of 0.97, indicating a definite linear relationship between amine concentration and detector response.

The recoveries of amines from the various extraction steps were also investigated (Table 2). Nine distinct samples of canned tuna (different brands and lot numbers) were used in this analysis. Methanol extraction of amines from swiss cheese was also investigated (Table 3). The extraction appeared to be somewhat less efficient

Table 1. Linear regression equations [a] and correlation coefficients for amine standards

Amines	Linear regression coefficients		Correlation coefficients
	a	*b*	
Histamine	0.0129	0.0195	1.00
Tyramine	0.0046	−0.0070	0.97
Putrescine	0.0173	0.0347	1.00
Cadaverine	0.0163	0.0557	1.00
Tryptamine	0.0029	0.0023	0.99
β-Phenylethylamine	0.0054	0.0226	0.99

[a] $Y = ax + b$, where Y = relative peak area and x = concn of amines in mg/100 g sample.

Table 2. Efficiencies of extraction from tuna

Amines	Av. recovery,[a] % ± SD		
	Methanol extn	Butanol–HCl extn	Overall extn
Histamine	90.7 ± 3.1	102.5 ± 2.8	92.93 ± 6.15
Tyramine	101.2 ± 6.9	93.4 ± 1.5	94.57 ± 6.34
Putrescine	90.7 ± 6.4	99.3 ± 1.8	90.10 ± 6.23
Cadaverine	98.0 ± 4.6	101.3 ± 2.4	99.31 ± 4.87
Tryptamine	91.3 ± 9.0	48.0 ± 2.2	43.83 ± 4.48
β-Phenylethylamine	98.5 ± 6.1	66.5 ± 2.3	65.51 ± 4.33

[a] Average value of 9 different canned tuna samples.

Table 4. Comparison of analytical values of histamine in swiss cheese

Sample	AOAC method, mg/100 g		HPLC method, mg/100 g
	1[a]	2[b]	
19-1	4.7	2.8	4.4
19-2	32.5	30.3	30.3
19-3	87.0	66.2	85.3

[a] AOAC method performed at FDA, Division of Food Technology.
[b] AOAC method performed at this laboratory.

and more variable. Therefore, extraction efficiencies should be determined for each type of food. With the sequential butanol–HCl extractions, good recoveries were obtained with most of the amines except tryptamine and β-phenylethylamine. Their poor recoveries were probably due to insufficient partition of these 2 amines in butanol and HCl phases. However, linear regression analysis of their standard curves yielded very high correlation coefficients. The recoveries of these 2 amines were low but constant at the various amine concentrations. Consequently, these poor recoveries do not pose a serious problem in the assay system, other than causing a decreased sensitivity at low concentrations.

In analysis of 9 samples of canned tuna by this method, the amines were present at nondetectable levels.

To study the application of this HPLC procedure to cheese products, 3 Swiss cheese samples were analyzed. Histamine contents were first determined at the FDA Division of Food Technology using AOAC method **18.067–18.071** (14). These samples were then sent to our laboratory, both AOAC and HPLC analysis were used, and results were compared (Table 4). Results obtained from the 3 analyses were similar. AOAC analysis of Sample 19-3 in our laboratory yielded a lower result than obtained by the other 2 methods. The basis for this discrepancy is unknown. Unfortunately, we did not have enough sample to repeat these analyses. A typical chromatogram of a cheese sample is shown (Figure 2). Cheese samples contained higher concentrations of amino acids than did tuna. Amine concentrations were calculated from the relative peak area (Table 5). All of the amines except tryptamine and β-phenylethylamine occurred naturally in Swiss cheese samples. Samples which had high histamine content also had high concentrations of other amines. It is known that decomposed fish and aged cheese have higher concentrations of amines (8, 9) but no clear relationship has been shown. This HPLC procedure for determination of amines can serve as a useful tool for further investigations.

Table 3. Efficiencies of methanol extraction of swiss cheese

Amines	Av. recovery,[a] % ± SD
Histamine	90.4 ± 12.0
Tyramine	102.3 ± 18.1
Putrescine	91.8 ± 11.5
Cadaverine	92.1 ± 11.1
Tryptamine	86.5 ± 6.8
β-Phenylethylamine	79.0 ± 15.9

[a] Average value of 8 different swiss cheese samples.

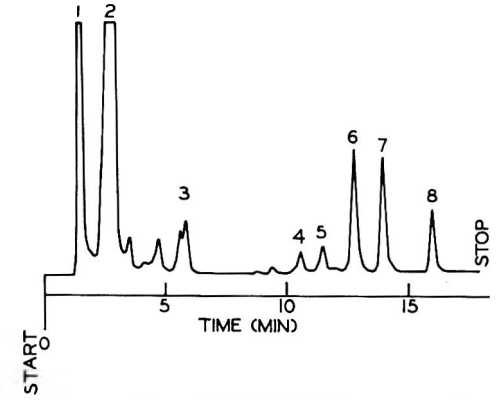

Figure 2. Chromatogram of dansylated amines from swiss cheese sample 19-3 by HPLC. 1 and 2, amino acids (unresolved); 3, 5-dimethylaminonaphthalene-1-sulfonic acid; 4, putrescine; 5, cadaverine; 6, histamine; 7, 1,7-diaminoheptane (internal standard); 8, tyramine.

Table 5. Amine content in swiss cheese determined by HPLC

Amines	Concn of amines, mg/100 g[a]		
	19-1	19-2	19-3
Histamine	4.4	30.3	85.3
Tyramine	ND[b]	45.0	98.8
Putrescine	ND	29.1	16.2
Cadaverine	ND	34.6	18.2
Tryptamine	ND	ND	ND
β-Phenylethylamine	ND	ND	ND

[a] Values calculated with extraction efficiencies taken into account.

[b] ND = not detectable.

In a survey of histamine levels in commercially processed scombroid fish products by Taylor et al. (16), over 90% of these products had histamine levels below 5 mg/100 g. The defect action level for histamine in scombroid fish products is 20 mg/100 g (17). This HPLC method is able to detect histamine at levels less than 5 mg/100 g. If increased sensitivity is needed, fluorescence detection can be used in place of UV detection (18). In a survey by Chambers and Staruszkiewicz (19) of a variety of cheese samples, histamine contents varied between different types of cheese, and ranged from below 1 mg/100 g to 250 mg/100 g. For the analysis of extremely high concentrations of amines (above 100 mg/100 g), sample dilution is suggested to improve the separation and sharpness of peaks. The best working range of amines with this method is below 100 mg/100 g. Depending on the sensitivity of instrument and type of foods analyzed, the lower limit of detection of these various amines was between 2 and 5 mg/100 g food sample.

This HPLC procedure for the simultaneous analysis of 6 putrefactive amines provides a useful tool for future studies of foodborne amines. The procedure has advantages in terms of specificity and accuracy. HPLC analysis can be automated by using an automated sampler. This reduces time and labor needed, and large numbers of samples can be analyzed in a relatively short period of time. This method should be a relatively simple way of assessing the levels of these chemical indicators of microbial spoilage in foods.

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DAIRY PRODUCTS

Compositional Analysis of Nonfat Dry Milk by Using Near Infrared Diffuse Reflectance Spectroscopy

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Proximate data from 82 nonfat dry milk (NFDM) samples were correlated with near infrared reflectance (NIR) measurements. The best wavelengths for determining constituent concentrations were chosen from 19 preselected filters by using linear regression analysis. The correlation coefficient (r) was 0.971 and the standard error of prediction (SEP) was 0.274 when the predicted values (from NIR measurements) using the 3 wavelengths selected for determining moisture content were compared with laboratory values; r and SEP were 0.961 and 0.099, respectively, when the predicted values using the 4 wavelengths selected for fat content were compared with laboratory results; 0.887 and 0.594, respectively, using the 4 wavelengths selected for lactose content; 0.905 and 0.438 using the 8 wavelengths selected for protein (micro-Kjeldahl) content; and 0.911 and 0.509 using the 7 wavelengths selected for protein (dye binding). These data indicate that NIR can be used to estimate moisture, fat, lactose, and protein content of NFDM.

Near infrared reflectance spectroscopy (NIR) is receiving widespread attention as a nondestructive method for rapidly measuring the composition of many products. NIR determines composition through the measurement of diffuse reflectance. Diffuse reflectance (DR) is light that has been transmitted through a portion of the product and, because of internal light scattering, emerges from the illuminated surface (1). DR is affected by the absorption and light-scattering properties of the product.

NIR has been successfully used to analyze protein and oil concentrations in corn, soybeans, and oats (2) and may be an effective method for evaluating forage quality (3, 4).

Goulden (5) reported the NIR spectra for casein, butter, dry whole milk, and lactose. High correlations with reflectance have been obtained for protein, fat, and moisture content in cheese, indicating the potential application of NIR for determining cheese composition (6).

The objective of this study was to measure the reliability of NIR in predicting moisture, fat, lactose, and protein content in NFDM. Data concerning the precision of NIR in determining NFDM composition could lead to its acceptance as a rapid, nondestructive analytical method.

Experimental

Samples

The composition of 82 NFDM samples was determined by analysis of both commercial and laboratory prepared samples.

(a) *Commercial samples.*—Thirty-five low heat, one medium heat, and 8 high heat NFDM samples were obtained from manufacturers located throughout the United States.

(b) *Laboratory prepared samples.*—Thirty-eight NFDM samples were prepared with elevated concentrations of different constituents. Ten NFDM samples were exposed to humidified air for various lengths of time to increase moisture content. These samples were sifted through a U.S.A. Standard Test Sieve No. 20 (850 mm opening) and mixed to ensure uniformity. Twenty-eight NFDM samples with increased concentrations of lactose, protein, or fat were prepared by spray-drying with an Anhydro Spray Drier 1967, Type Lab. S 1 (Anhydro Inc., Attleboro Falls, MA 02763). Reagent grade lactose monohydrate powder (J. T. Baker Chemical Co., Phillipsburg, NJ 08865) was added to 8 samples to raise the lactose content 1.5, 2.5, 3.5, and 4.5%. Buttermilk powder (Land O'Lakes, Inc., Minneapolis, MN 55413) was added to 8 samples to raise the butterfat content 0.7, 0.9, 1.1, and 1.3%. Certified casein powder (dissolved in 50°C distilled water with the pH adjusted to 10.0 with 29.6% ammonium hydroxide) and 80% whey protein concentrate were added to 8 samples to increase the protein content 1.5, 2.5, 3.5, and 4.5%. The casein and whey protein were added at levels that approximate those in milk protein (normally 80% casein and 20% whey

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Table 1. Composition (%) of nonfat dry milk samples

Constituent	No. samples for calibration	No. samples for prediction	$\bar{X}$[a]	Range
Moisture	24	19	4.36	2.85–9.70
Protein (dry wt)[b]	27	22	36.52	34.00–39.86
Protein (dry wt)[c]	26	22	37.08	35.54–40.68
Fat (dry wt)	23	18	0.96	0.52–2.07
Lactose (dry wt)	21	18	54.01	52.43–58.08

[a] Mean of duplicate or triplicate determinations.
[b] Protein by micro-Kjeldahl (nitrogen × 6.38 = protein).
[c] Protein by acid orange dye binding.

proteins). Four reconstituted NFDM samples with unaltered constituent concentrations were spray-dried in laboratory equipment and served as controls for this group of samples. All of these 28 samples were reconstituted to at least 20% total solids (dry weight) before spray-drying.

Methods

(a) *Compositional analysis.*—NFDM samples were analyzed, in duplicate or triplicate, for the following components using the methods indicated: moisture—toluene distillation method (7); nitrogen—micro-Kjeldahl method, secs. **34.021, 47.021** (8); protein—acid orange dye binding method, secs. **16.037-16.041** (8); fat—Mojonnier extraction test (9), modified to use 6.0 mL ammonium hydroxide instead of recommended 1.5 mL. An enzymatic-cryoscopic method (10) was used to measure lactose content. Samples were prepared for lactose analysis by rehydration to 9.0% total solids (dry weight). Lactase (β-D-galactosidase) was added and samples were incubated 2 h at 37°C to hydrolyze the lactose. Freezing point depression resulting from hydrolysis of lactose solutions of 4.00, 4.50, 4.75, 5.00, and 5.25% were determined for the standard curve. Lactose content for samples was calculated by using linear regression analysis.

(b) *Spectral analysis.*—Reflectance measurements were obtained by using a Technicon InfraAlyzer™ 400R (Technicon Industrial Systems, Tarrytown, NY 10591) equipped with 19 preselected filters. NFDM samples were placed in a Technicon sample holder which pressed the powder firmly against a glass window. Samples were analyzed in triplicate at 21°C.

(c) *Data analysis.*—Reflectance data were analyzed by using a multiple linear regression program. Samples selected for calibration and prediction of each constituent represented the complete range of constituent concentrations available. Reflectance data from the calibration samples were used to select a combination of filters whose reflectance values had the lowest standard error of estimate (SEE) and the highest multiple correlation coefficient (r_1) for each constituent. The standard error of prediction (SEP) and the correlation coefficient (r_2) were obtained for the calibrations for moisture, fat, lactose, and protein content.

Results

The numbers of NFDM samples used for the calibration and prediction of each constituent are shown in Table 1. As expected, constituent levels for some experimentally prepared samples were substantially above normal concentrations in NFDM (Table 1). It was necessary to work with a wide range of constituent concentrations to assure that the filters selected directly measure the level of each constituent rather than producing results based on intercorrelations between constituents.

Table 2. Absorption bands of NFDM constituents

Filter No.[a]	Absorption band[b] (nm)	Constituents contributing to absorption[c]
2	2340	fat, casein, lactose
4	2310	fat, casein
5	2270	fat, casein
8	2190	casein
11	1980	casein, water
12	1820	casein, lactose (fat reference)
13	1780	(fat, casein reference)
14	2100	casein, lactose (water reference)
16	1940	water
17	1730	fat, casein
18	1720	fat, casein
19	1450	casein, lactose, water
20	1680	casein, (water reference)

[a] Filters of the Technicon NIR analyzer which correspond to the given wavelength bands.
[b] Approximate wavelength.
[c] From Goulden (5), and Frank and Birth (6).

Table 3. Near infrared reflectance data for moisture, fat, and lactose in nonfat dry milk

Filter No.	Constant[a]	r_1[b]	r_2[c]	SEE[d]	SEP[e]
		Moisture			
	2.388	0.980	0.971	0.217	0.274
11	75.607				
14	−54.423				
16	−1.889				
	2.892	0.981	0.971	0.216	0.274
11	76.458				
14	−45.874				
20	−21.974				
		Fat (Dry Wt)			
	0.207	0.959	0.961	0.081	0.099
4	292.097				
5	−319.695				
12	−66.767				
18	105.499				
		Lactose (Dry Wt)			
	58.477	0.858	0.887	0.483	0.594
2	224.350				
13	1226.560				
17	−1924.921				
19	348.398				

[a] % Constituent = $K_0 + K_1 (\log 1/R_1) + K_2 (\log 1/R_2) \ldots$.
[b] Multiple correlation coefficient for calibration.
[c] Correlation coefficient for all samples.
[d] Standard error of estimate.
[e] Standard error of prediction.

Table 2 lists the wavelength bands of the filters used and the milk constituents which absorb at these wavelengths. The precise wavelength of each filter is proprietary information of Technicon.

NIR calibration and prediction data are presented in Tables 3 and 4. Constants obtained using the formula, % constituent = $K_0 + K_1 (\log 1/R_1) + K_2 (\log 1/R_2) + \ldots$, are listed for each calibration (K_0 = intercept or bias; K_n = constants

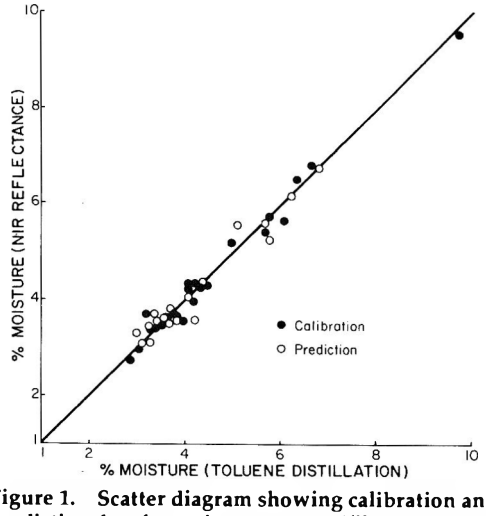

Figure 1. Scatter diagram showing calibration and prediction data for moisture content (filters 11, 14, 16) by NIR.

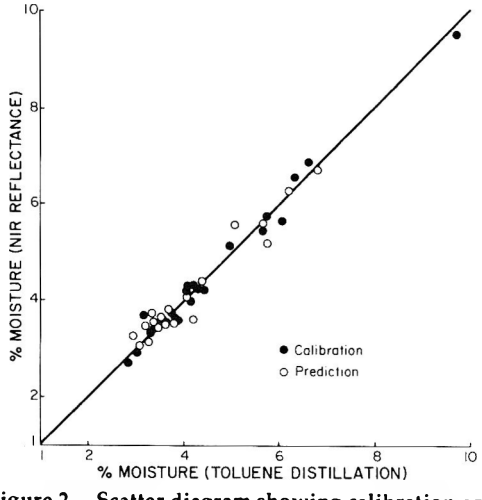

Figure 2. Scatter diagram showing calibration and prediction data for moisture content (filters 11, 14, 20) by NIR.

Table 4. Near infrared reflectance data for protein (dry wt) in nonfat dry milk

Filter No.	Constant[a]	r_1[b]	r_2[c]	SEE[d]	SEP[e]
		Micro-Kjeldahl Method[f]			
	36.260	0.957	0.905	0.254	0.438
4	1050.875				
5	−2337.304				
8	1367.231				
11	117.003				
12	2112.350				
13	−3804.322				
17	1848.267				
19	−367.735				
		Acid Orange Dye Binding Method			
	32.537	0.895	0.911	0.352	0.509
5	−693.250				
8	801.840				
11	109.015				
12	2021.491				
13	−3130.019				
17	1409.839				
19	−571.359				

[a–e] See Table 3.
[f] Nitrogen × 6.38 = protein.

for corresponding filters; R_n = reflectance values).

Discussion

Figures 1–6 illustrate calibration and prediction data for all constituents. These data indicate that prediction of all constituents by NIR is linear throughout the range tested.

Data of Frank and Birth (6) and Goulden (5) indicate extensive overlapping between NIR absorption bands of major milk constituents (Table 2). This is typical of NIR spectra of agricultural commodities. To measure the level of a particular constituent, filters are selected which correspond to wavelengths at which absorption correlates with constituent concentration and additional (reference) wavelengths which counteract interference from other constituents. Absorbance measurements at 2 to 4 wavelengths are usually necessary to adequately predict the concentration of a particular constituent. The complexity of NIR spectra requires the use of

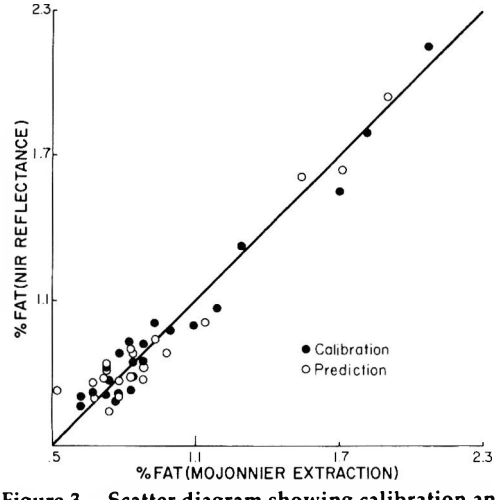

Figure 3. Scatter diagram showing calibration and prediction data for fat content by NIR.

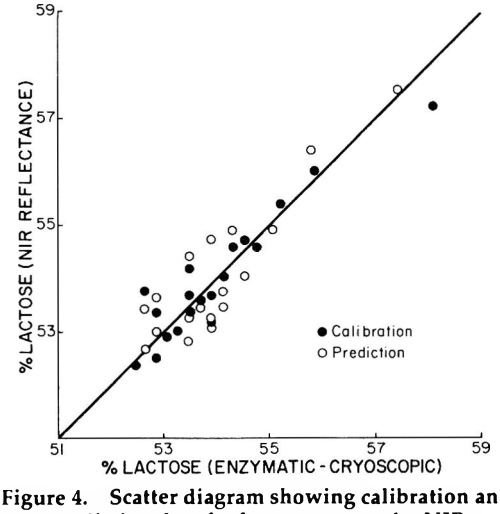

Figure 4. Scatter diagram showing calibration and prediction data for lactose content by NIR.

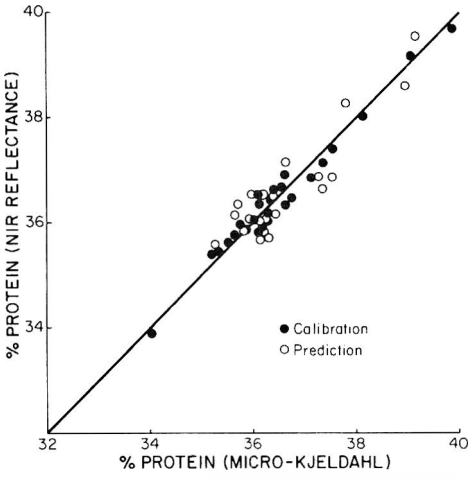

Figure 5. Scatter diagram showing calibration and prediction data for protein content (micro-Kjeldahl) by NIR.

multiple linear regression analysis to select predictive wavelengths. The NIR instrument used in this study was equipped with 19 filters selected to include absorption bands of significance in analyzing the composition of agricultural commodities.

Calibration data for 2 sets of filters are included for moisture analysis (Table 3) because each set exhibited nearly equivalent predictive ability. Water has significant absorption peaks at 2 of the filters used for prediction (filters 11 and 16). Of the 4 filters selected to predict fat content, filters 4, 5, and 18 correspond to absorption peaks. Filters 2, 13, 17, and 19 were selected for lactose measurement. Lactose has absorption bands at wavelengths measured by filters 2 and 19 (Table 3), according to the reflectance spectrum reported by Goulden (5).

Eight filters were selected for protein (micro-Kjeldahl) prediction. Filters 4, 5, 8, 11, 12, 17, and 19 correspond to areas of casein absorption bands. With the exception of filter 4, the same filters were selected for predicting protein by the dye binding method. The increased number of filters necessary for protein prediction may be due to a partial masking of protein absorption by lactose (the major NIR absorbing component of NFDM) in wavelength bands at which the protein measuring filters are located. A greater selection of filters (other than the 19 filters with which the instrument was equipped) for protein absorption bands might permit the use of fewer filters to obtain an adequate prediction.

Since the NFDM samples used in this study originated at various plants, they varied in particle size and degree of heat treatment, as well as composition. Each of these factors has an effect on NIR. Our results indicate that usable predictions of composition can be obtained even when physical factors such as particle size are not constant. It is possible that NIR calibrations at individual dairy plants will result in lower SEP values because of the greater physical homogeneity of samples.

This study indicates that NIR instrumentation can be used to estimate moisture, fat, lactose, and protein content of NFDM.

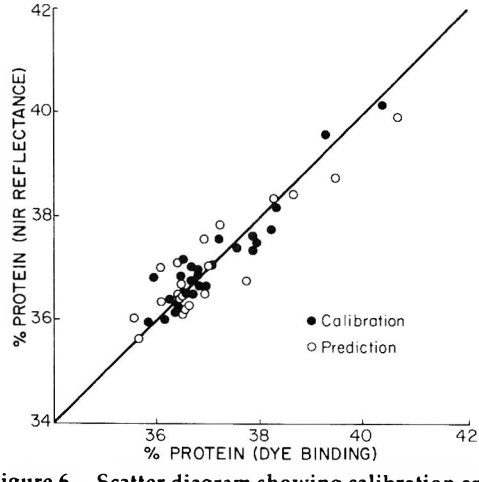

Figure 6. Scatter diagram showing calibration and prediction data for protein content (dye binding) by NIR.

Acknowledgments

The authors thank Technicon Industrial Systems, Tarrytown, NY, and the NFDM manufacturers for providing materials used in this study; V. N. M. Rao and W. T. Haley of the Food Science Department, University of Georgia, for their assistance in spray-drying samples; and C. J. Conde, M. R. Furnia, B. J. Goochee, C. M. Pericak, and P. L. Smith for technical assistance.

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DRUGS

High Pressure Liquid Chromatographic Determination of Oxazepam Dosage Forms: Collaborative Study

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A reverse phase high pressure liquid chromatographic method for the determination of oxazepam in tablets and capsules was collaboratively studied by 9 laboratories. Collaborators were supplied with 6 samples that included synthetic and commercial formulations. Tablet and capsule composites are diluted with methanol and filtered. Oxazepam is determined at 254 nm by using a C_{18} column. Mean recoveries of oxazepam from synthetic tablet and capsule formulations were 97.2 and 99.0%, respectively. Mean coefficients of variation for tablets and capsules ranged from 1.85 to 2.86%. The method has been adopted official first action.

Oxazepam is a psychotropic agent in the benzodiazepine class which is useful in the management and control of anxiety, tension, agitation, irritability, and related symptoms commonly seen in patients with a diagnosis of psychoneurotic or psychophysiological reaction. Oxazepam is structurally related to chlordiazepoxide and diazepam, and the relative effectiveness of these compounds as tranquilizing agents has been reported (1).

Reviews by Clifford and Smyth (2), Hailey (3), and Shearer and Pilla (4) list methods for the determination of oxazepam and other benzodiazepines in biological fluids by polarographic, gas-liquid chromatographic, UV spectrophotometric, and liquid chromatographic techniques. There are numerous other references for the high pressure liquid chromatographic (HPLC) determination of oxazepam and its metabolites and hydrolysis products that are of clinical and forensic interest (5–8).

An accurate method was recently developed for the determination of oxazepam in pharmaceutical dosage forms (9). A reverse phase HPLC system using octadecyl-bonded silica microparticles as the stationary phase is employed. The mobile phase consists of methanol–water–glacial acetic acid (60 + 40 + 1). This system separates oxazepam from its degradation products as well as from impurities that may be present. The sample is diluted with methanol and filtered. The results of a collaborative study of the method are reported here.

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The recommendation of the Associate Referee was approved by the General Referee and Committee B and was adopted by the Association. See the reports of the General Referee and Committee B in *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

Oxazepam in Tablets and Capsules Liquid Chromatographic Method Official First Action

40.D01 ***Principle***

Oxazepam is extd into MeOH and detd by liq. chromatogy with UV (254 nm) detector.

40.D02 ***Apparatus***

(Equiv. ap. may be substituted)

(**a**) *Liquid chromatograph.*—Model 204 equipped with 2 Model 6000A pumps, Model 660 solv. programmer, Model 440 UV (254 nm) detector, Model U6K injector (all Waters Associates, Inc.), and Model 3380A integrator (Hewlett-Packard).

(**b**) *HPLC column.*—Bondapak C_{18}, 3.9 mm id × 30 cm long (Waters Associates, Inc.) at ambient temp.

(**c**) *Filter.*—Millipore type EG (pore size 0.2 μm) (Millipore Corp., Bedford, MA 01730) or 0.45 μm MeOH compatible equiv.

40.D03 ***Reagents***

(**a**) *Methanol.*—UV grade (MCB Reagents), or equiv.

(**b**) *Mobile phase.*—MeOH-H_2O-glac. HOAc (60 + 40 + 1) at flow rate of 1.0 mL/min. MeOH concn and flow rate may be varied to give approx. retention time of 6–8 min for oxazepam.

(**c**) *System suitability std soln.*—Dissolve 10 mg

USP Oxazepam and 15 mg USP 2-Amino-5-chlorobenzophenone in 250.0 mL MeOH.

(d) *Oxazepam std soln.*—Transfer 25 mg USP Oxazepam, accurately weighed, to 250 mL vol. flask. Add 5 mL H_2O and dil. to vol. with MeOH. Soln is stable 90 min.

40.D04 *Sample Preparation*

(a) *Tablets.*—Det. av. wt/tablet and grind tablets to pass No. 60 mesh sieve. Transfer accurately weighed portion of powder contg 25 mg oxazepam to 250 mL vol. flask. Add 5 mL H_2O and 25 mL MeOH. Mix thoroly. Add 75 mL MeOH and place in ultrasonic bath 10 min. Dil. to vol. with MeOH. Stir 30 min. Filter portion of soln thru type EG filter into small g-s flask. Soln is stable 90 min.

(b) *Capsules.*—Det. av. wt of capsule contents. Transfer accurately weighed portion of capsule contents contg 25 mg oxazepam to 250 mL vol. flask. Proceed as in (a).

40.D05 *System Suitability Check*

(a) *Resolution.*—Inject 10.0 μL system suitability std soln. Retention times for oxazepam and 2-amino-5-chlorobenzophenone should be ca 6 and 14 min, resp. Resolution factor, R, for the 2 peaks should be ≥5.0, using following formula:

$$R = 2(t' - t)/(PW + PW')$$

where t and t' = mm retention of oxazepam and 2-amino-5-chlorobenzophenone peaks, resp.; and PW and PW' = mm peak widths measured at baseline of oxazepam and 2-amino-5-chlorobenzophenone, resp. Adjust MeOH concn if resolution is unsatisfactory.

(b) *Reproducibility.*—Make five 10.0 μL injections of oxazepam std soln and measure peak areas. In suitable system, coefficient of variation is not >2.0%.

40.D06 *Determination*

Make duplicate 10 μL injections each of sample soln and std soln, alternating sample and std solns. Calc. results by using peak areas:

$$\text{mg Oxazepam/tablet or capsule} = (PA/PA') \times C \times (T/S)$$

where PA and PA' = peak area for sample and std solns, resp.; C = mg oxazepam/250 mL std soln;

Table 1. Results (mg) of collaborative study for oxazepam

	Sample					
Coll.	1	2	3	4	5[a]	6[b]
1	10.0	14.4	14.6	29.3	14.0	9.5
	9.4	14.5	14.5	28.3	13.9	9.3
2	9.7	14.6	14.6	28.7	14.2	9.9
	9.8	14.6	14.4	29.2	14.5	10.1
3	9.8	15.2	14.7	28.2	14.0	9.4
	10.0	15.0	14.8	28.3	14.0	9.9
4	9.9	14.8	14.8	28.8	13.6	9.2
	9.9	15.0	14.8	28.1	14.5	9.7
5	9.7	15.2	14.9	29.8	14.4	9.8
	9.6	15.0	15.4	29.7	14.1	9.7
6[c]	10.1	15.2	15.0	25.3	15.2	10.5
	10.0	15.2	15.0	27.3	14.9	10.5
7	9.5	14.5	14.6	28.8	14.3	9.9
	9.5	14.6	14.5	28.5	14.2	9.7
8	9.0	14.5	14.6	28.7	14.2	9.7
	9.3	14.7	14.7	28.3	14.1	9.6
9	9.9	14.8	15.2	29.8	14.2	10.0
	9.8	15.2	15.1	30.8	13.6	10.0
Mean	9.7	14.8	14.8	29.0	14.1	9.7
Rec., %	97.0	98.7	98.7	96.7	97.2	99.0
Range						
Low	9.3	14.4	14.4	28.1	13.6	9.3
High	10.0	15.2	15.2	30.8	14.5	10.1
SD	0.277	0.278	0.273	0.754	0.266	0.260
CV, %	2.86	1.88	1.85	2.60	1.88	2.68

[a] 14.8 mg synthetic formulation.
[b] 9.8 mg synthetic formulation.
[c] Mean, SD, and CV do not include Collaborator 6.

Table 2. Results of system suitability test for oxazepam

Coll.	Column	CV, %	Resolution	Retention, min (Oxazepam/2-amino-5-chlorobenzophenone)
1	Micropak C-18	0.42	8.71	8.16/20.52
2	Zorbax ODS	0.74	8.5	5.85/12.85
3	Ultrasphere ODS	1.04	8.6	7.70/19.43
4	Alltech C-18	1.14	11.1	10.8/29.8
5	Zorbax ODS	1.1	15.7	5.35/12.56
6[a]	Zorbax BP-ODS	3.7	7.9	5.20/12.13
7	Chromegabond C-18	0.68	10.7	6.40/20.8
8	Spherisorb ODS	1.1	6.7	3.25/7.09
9	Bondapak C_{18}	0.55	26.2	7.53/16.72

[a] Did not meet system suitability test.

T = av. wt, g, of tablet or capsule contents; S = sample wt, g.

Collaborative Study

A collaborative study was conducted with 9 collaborators. The samples consisted of 4 capsule formulations (3 commercial, 1 synthetic) and 2 tablet formulations (1 commercial, 1 synthetic). Each collaborator was supplied with 1 g each of the 6 samples, 600 mg oxazepam standard, and 30 mg 2-amino-5-chlorobenzophenone standard. Each collaborator received instructions, a copy of the method, and a reporting form from the Associate Referee.

Results and Discussion

Individual results from each collaborator are shown in Table 1 along with the mean, standard deviation, range, and coefficient of variation for each of the 6 samples. The results from Collaborator 6 could not be used because the system suitability test for reproducibility was not met. Table 2 lists the column and system suitability information received from each collaborator.

Collaborators 1, 2, and 8 filtered their solutions before injection. Collaborator 2 recommended that the solutions be filtered to protect the HPLC system. A filtering step has been added to the method. No adverse comments concerning the methodology were received.

Recommendation

It is recommended that this method for the determination of oxazepam in tablets and capsules be adopted official first action.

Acknowledgments

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Identification and Estimation of the Alkaloids of *Rauwolfia serpentina* by High Performance Liquid Chromatography and Thin Layer Chromatography

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A procedure is presented for the identification and estimation of some of the alkaloids of *Rauwolfia serpentina* by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Two extraction procedures were studied, one essentially similar to the current AOAC method, and one using warm methanol. For HPLC, a μPorasil column, methanol as eluting solvent, and a fluorometric detector were used. Rescinnamine was detected at 330 nm, at which wavelength reserpine fluorescence is negligible. Reserpine was detected at 280 nm, where rescinnamine fluorescence is small. Other alkaloids detected were raubasinine, ajmalicine, yohimbine, ajmaline, and serpentine. For TLC, $CHCl_3$-CH_3OH (97 + 3) and $CHCl_3$-CH_3OH (80 + 20) were used as developing solvents and spots were detected under long- and shortwave UV light. A semiquantitative TLC procedure was also developed for serpentine, the content of which was found to be in the 0.2-0.25% range.

Preparations containing the ground root of *Rauwolfia serpentina* are indicated for the treatment of hypertension and mental disorders (1). Other potential but less common uses are in the field of geriatrics and in the treatment of psoriasis, angina, constitutional leanness, and gynecological disorders (1).

The chemical structure has been extensively investigated and several compounds have been isolated. The review by Phillips and Chadha (1) lists 25 alkaloids, including ajmaline, ajmalicine, deserpidine, raubasinine (reserpinine), reserpine, rescinnamine (reserpinine), reserpiline, and serpentine. The term reserpinine has been used by some authors as a synonym of rescinnamine ($C_{35}H_{42}O_9N_2$) and by some others as a synonym of raubasinine ($C_{22}H_{26}O_4N_2$). It is believed that the hypotensive action of *Rauwolfia* is due primarily to the weakly basic alkaloids deserpidine, reserpine, and rescinnamine; the functions, if any, of the other compounds are a matter of controversy (1).

The current USP method for the analysis of *Rauwolfia serpentina* preparations (2) is essentially that of Banes et al. (3). The weakly basic alkaloids are quantitatively extracted and, in the process, as much as possible of the other compounds is removed. For quantitative determination, the purified solutions are reacted with sodium nitrite according to the procedure of Szalkowski and Mader (4), and the absorbances of the reaction products are measured at 390 nm. Reserpine and rescinnamine react with approximately equal intensity (3); sarpagine, yohimbine, and deserpine do not react (4, 5). The sample solution, despite the extensive purification, contains trace quantities of other alkaloids that react similarly to reserpine. Haycock and Mader (5) identified 2 such compounds as raubasinine (reserpinine) and reserpiline, but there are probably others.

The USP requirements of 0.15-0.20% reserpine-rescinnamine group alkaloids take into account the contribution of other alkaloids. In a subsequent publication, Banes et al. (6), using column chromatography and UV spectrophotometry, estimated that the other alkaloids increase the reserpine-rescinnamine content by a factor of 1.11-1.18. These authors (6) also presented a method for estimating deserpidine, which is not determined by the USP procedure. The deserpidine content of some samples of *Rauwolfia serpentina* by this method (6) was 3-6 mg per 100 g (0.003-0.006%).

The USP method (2) requires a large sample, is lengthy, and is difficult to adapt to single tablet assay. A shortened version of the USP method was published by Kunze et al. (7). The advent of spectrofluorometry as an analytical tool permitted still further simplifications. The procedure developed by Clark (8) was an attempt to achieve the same results as the USP method on a smaller scale; that is, the weakly basic alkaloids were isolated and then reacted with sodium nitrite in acid medium. This fluorometric method was later collaboratively studied (9), but the standard deviation was considered to be rather high. A new collaborative study was conducted in which vanadium pentoxide replaced sodium nitrite as the chromogenic reagent (10), according to the procedure of Urbanyi and Stober (11).

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The results were considered satisfactory and the method was adopted as official by AOAC (12).

The main purpose of the present investigation was to identify the alkaloids extracted by the current AOAC method and by warm methanol. Another goal was to examine the feasibility of making determinations by high performance liquid chromatography (HPLC). Several *Rauwolfia* alkaloids have been chromatographed by HPLC using a fluorescent detector (13), although the problem of sample analysis was not specifically considered.

Experimental

Apparatus

(a) *Fluorescence spectrophotometer.*—Perkin-Elmer Model MPF-3.

(b) *Liquid chromatograph.*—Model U6K universal injector and Model 6000A solvent delivery system (Waters Associates, Milford, MA 01757) connected to FS-970 fluorescence detector (Kratos Analytical Inst., Westwood, NJ 07675).

(c) *HPLC column.*—μPorasil, 30 cm long by 3.9 mm id (Waters Associates) or equivalent direct phase column.

(d) *Preparative TLC plates.*—Slurry 60 g silica gel H (Brinkmann Instruments, Westbury, NY 11590) with 140 mL water, coat five 20 × 20 cm plates to thickness of 0.5 mm with the slurry, and dry in air.

(e) *TLC plates.*—Slurry 30 g silica gel H and 100 mg F254 green fluorescent indicator (Brinkmann Instruments) with 70 mL water, coat five 20 × 20 cm plates to thickness of 0.25 mm with the slurry, and dry in air.

(f) *Prepared column.*—Plug constricted end of 30 × 2 cm id glass column with cotton. Mix 3 g Celite 545 (Fisher Scientific Co., Fairlawn, NJ 07410) with 2 mL 0.1N NaOH, and transfer to column. Compact with rod, and overlay with pledget of cotton.

Reagents

(a) *Methanol.*—HPLC grade (Burdick & Jackson, Muskegon, MI 49442).

(b) *Sulfuric acid solution.*—0.5N H_2SO_4. Dilute 14 mL H_2SO_4 to 1 L in H_2O, mix well, and cool to room temperature.

(c) *Rauwolfia alkaloids.*—Ajmalicine HCl (AJC), ajmaline (AJL), deserpidine (DSP), yohimbine HCl (YO), reserpine (RS), rescinnamine (RC), reserpiline oxalate (RPL), serpentine bitartrate (SPT) (ICN K&K Labs., Plainview, NY 11803).

(d) *Rauwolfia serpentina powder.*—USP; available from U.S. Pharmacopeial Convention, Rockville, MD 20852 and Pfaltz & Bauer, Stamford, CT 06902.

(e) *Aricine (AR) and sarpagine (SPG).*—Sandoz Pharmaceuticals, East Hanover, NJ 07936. Small quantities were kindly provided by John W. Robinson, FDA, New York District.

HPLC Solutions

(a) *Reserpine solutions.*—(*1*) *Stock solution:* Transfer 50 mg reserpine to 100 mL volumetric flask, add 1 mL $CHCl_3$, swirl well to dissolve, add methanol to mark, and mix. (*2*) *Intermediate solution:* Dilute 10.0 mL reserpine stock solution to 100.0 mL with methanol. (*3*) *0.25 solution:* Dilute 5.0 mL reserpine intermediate solution to 100 mL with methanol.

(b) *Rescinnamine solutions.*—(*1*) *Stock solution:* Transfer 30 mg rescinnamine to 100 mL volumetric flask, add 1 mL $CHCl_3$, swirl well to dissolve, add methanol to mark, and mix. (*2*) *Intermediate solution:* Dilute 10.0 mL rescinnamine stock solution (*1*) to 100.0 mL with methanol. (*3*) *0.30 solution:* Dilute 10.0 mL rescinnamine intermediate solution (*2*) to 100.0 mL with methanol. (*4*) *0.060 solution:* Dilute 2.0 mL rescinnamine intermediate solution to 100.0 mL with methanol.

(c) *Mixed reference solution.*—Transfer 4.0 mL reserpine intermediate solution (**a**) (*2*) and 2.0 mL rescinnamine intermediate solution (**b**) (*2*) to 100 mL volumetric flask, add methanol to mark, and mix. Solution contains 0.20 mg RS and 0.060 mg RC.

(d) *Sample extract.*—(*1*) *Rauwolfia serpentina powder:* Transfer 100 mg *Rauwolfia serpentina* to 250 mL separator containing 6 mL methanol, stopper, and shake well 1 min. Add 60 mL 0.5N H_2SO_4, stopper, and mix well; then extract with 30 mL $CHCl_3$. After layers have separated, transfer extract to prepared column (**f**), receiving eluate in 150 mL beaker. Extract with 3 additional 30 mL portions of $CHCl_3$, adding each to column. Add 10 mL methanol to beaker and heat on steam both with aid of air current until volume is reduced to ca 10 mL (do not evaporate to dryness). Add 30 mL methanol and continue heating until volume is reduced to ca 15 mL. Transfer solution to 50 mL volumetric flask, rinse beaker with small portions of methanol, add methanol to mark, and mix.

(*2*) *Rauwolfia serpentina tablets (ground composition):* Weigh 20 tablets, determine average weight, and grind in mortar to uniform powder. Transfer weighed amount of ground sample equivalent to ca 1 tablet to 250 mL separator containing 6 mL methanol; then continue as for

powder. If the label declaration is 50 mg, make the following changes: Evaporate solution in 150 mL beaker to 10 mL (rather than 20); then transfer to 25 mL volumetric flask (rather than 50).

(**e**) *Sample methanol solution.*—Transfer ca 100 mg *Rauwolfia serpentina* to 50 mL volumetric flask, add 20 mL methanol, and warm on steam bath 10 min. Cool to room temperature, add methanol to mark, mix, and filter through paper.

HPLC Determination

Set excitation at 280 nm and insert 370 nm filter. Elute methanol through column at flow of 1.5 mL/min. Inject 50 μL reserpine 0.25 solution (**a**) (*3*). Adjust flow rate, if necessary, so that reserpine peak appears after 4–5 min. Adjust sensitivity so that height of reserpine peak is ca 85% of chart height. Inject 50 μL sample extract (**d**). The chromatogram should include, in addition to the reserpine peak, another peak ca 2 min earlier. After above conditions have been met, proceed as follows: Inject two 50 μL aliquots each of reserpine 0.25 solution (**a**) (*3*), rescinnamine 0.30 solution (**b**) (*3*), and sample extract (**d**). Inject 40 μL sample methanol solution (e) and let chromatogram run ca 4 h. Then inject with 15 μL aliquot and let chromatogram run 20 min.

Change excitation to 330 nm but keep 370 nm filter and maintain same flow. Increase sensitivity 25 times; then inject 50 μL rescinnamine 0.060 solution (**b**) (*4*). Adjust sensitivity if necessary, so that RC peak height is 60–80% of chart height. Inject two 50 μL portions each of rescinnamine 0.060 solution (**b**) (*4*), mixed reference solution (**c**), and sample extract (**d**).

TLC Identification Tests

TLC identification solutions.—(*1*) U1: Transfer ca 2.5 mg RS, 5 mg AJC, and 5 mg DSP to 125 mL glass-stopper flask and add 50 mL methanol. Warm on steam bath to dissolve, cool to room temperature, add methanol to 100 mL, and mix. (*2*) U2: Transfer ca 2.5 mg SPT and 10 mg each of AJL and YO to 125 mL glass-stopper flask and continue as under U1.

Test solution 1.—After HPLC determinations have been completed, transfer 10 mL sample extract (**d**) to small flask and evaporate to dryness on steam bath with aid of air current. To residue, add 1.0 mL methanol, warm briefly on steam bath, and swirl.

Test solution 2.—Transfer weighed amount of sample equivalent to ca 20 mg *Rauwolfia serpentina* to small beaker, add 5 mL methanol, and warm 4–5 min on steam bath while stirring. Filter through paper, receiving filtrates in small flask, and evaporate to dryness over steam bath. Add 1.0 mL methanol to residue, warm briefly on steam bath, and swirl.

Using a small disposable pipet, repeatedly spot ca 100 μL TLC identification solution U1 on TLC plate (**e**). On same plate, spot ca 100 μL each of test solution 1 and test solution 2. Develop in $CHCl_3$–CH_3OH (97 + 3) to height of ca 15 cm, remove from tank, dry in hood, and then view under long- and shortwave UV light.

On another plate, spot ca 100 μL each of TLC identification solution U2 and test solutions 1 and 2. Develop in $CHCl_3$–CH_3OH (80 + 20) to height of 15 cm, remove from tank, dry in hood, and then view under long- and shortwave UV light.

Semiquantitative Estimation of Serpentine by TLC

Serpentine solution.—Transfer ca 4 mg serpentine bitartrate, accurately weighed on electrobalance, to 100 mL volumetric flask and add 40 mL methanol. Warm on steam bath to dissolve, cool to room temperature, add methanol to mark, and mix.

Sample solution.—Transfer weighed amount of sample equivalent to ca 100 mg *Rauwolfia serpentina* to 10 mL volumetric flask, add 5 mL methanol, and warm 5 min on steam bath. Cool to room temperature, add methanol to mark, mix, and filter through paper. Spot four 100.0 μL aliquots each of serpentine solution and sample solution, using 2 TLC plates (**e**). Develop in $CHCl_3$–CH_3OH (80 + 20) to height of 15 cm.

View dried plates under longwave UV light and circle blue serpentine spots. Scrape each spot with suction device, transfer to small glass-stopper flask, add 10.0 mL methanol, stopper, shake well 1 min, and filter through paper. Set excitation at 370 nm on fluorescence spectrophotometer (**a**), transfer one of the filtered extracts of the reference to 1 cm cell, and then adjust sensitivity so that emission peak around 440 nm is ca 70% of chart height. Scan all filtered extracts in the emission mode from 300 to 600 nm. Average the maxima of the 4 reference extracts. If one or more of the values differ from average by more than 15%, discard the one that has the greatest deviation and then recalculate average. Proceed similarly with readings of sample extract. Designate average maximum of reference as Fr and average maximum of sample as Fs. Calculate amount of serpentine in sample solution by formula

Serpentine (mg)

$$= (Fs)(Wr)(0.1)(348.39)/(Fr)(498.49)$$

where Wr indicates mg of serpentine bitartrate in 100 mL reference solution.

Identification of Other Alkaloids

The following procedure was carried out in an attempt to identify unknown compounds A, B, and U: Transfer 50 g *Rauwolfia serpentina* to 1 L separator containing 100 mL 0.1N H_2SO_4, extract with 4 successive 500 mL portions of $CHCl_3$, and filter extracts through cotton, receiving filtrate in 2 L beaker marked AB. Add 40 mL 1N NaOH to separator, mix, and extract with four 500 mL portions of $CHCl_3$, receiving extract in 2 L beaker marked U. Extract another 50 g portion of *Rauwolfia serpentina* in a similar manner.

Evaporate $CHCl_3$ in each beaker to ca 50 mL over steam bath with aid of air current. Combine 2 AB solutions in 250 mL beaker, evaporate to ca 10 mL, transfer to 25 mL flask, and rinse beaker with small portions of $CHCl_3$. Evaporate to dryness, add 4 mL $CHCl_3$, and swirl well to dissolve residue. Spot 1 mL across entire length of preparative TLC plate (**d**). Spot remaining solution on 3 other plates. Develop plates in $CHCl_3$–CH_3OH (97 + 3) to height of 15 cm, dry, and view under longwave UV light. Circle blue bands in upper sections of plates, without including much of green bands immediately below; scrape and combine in 150 mL beaker marked B.

Extract adsorbent with 4 successive 25 mL portions of methanol, and filter through paper, receiving filtrates in 150 mL beaker. Evaporate to dryness over steam bath, dissolve in small volume of methanol, spot on preparative TLC plates (**d**), and develop in $CHCl_3$–CH_3OH (97 + 3). Proceed similarly with green fluorescent bands (identify as A).

Continue chromatographic purifications of A and B until they are completely or almost completely free of each other. Concentrate final methanol extract of A to 3 mL, transfer to tared 5 mL beaker, evaporate methanol, dry beaker at 100°C for 1 h, cool, and reweigh. (Residue was only about 2.5 mg; consequently weight is not very accurate.) Dissolve residue and dilute to 100 mL with methanol. Scan UV absorbance in 1 cm cell from 350 nm to 200 nm. Transfer two 5.0 mL aliquots to separate 10 mL volumetric flasks, react one aliquot with sodium nitrite in acid medium as in (9), and use the other as blank. Examine also by TLC and HPLC, using the conditions described above. Follow the same procedure for Compound B.

Evaporate solutions in beaker U, dissolve residue in 4 mL $CHCl_3$, spot on preparative TLC plates, and develop in $CHCl_3$–CH_3OH (80 + 20) (see Figure 1). Under longwave UV light, circle most intense green bands around the middle of the plates. Scrape circled bands, extract and purify as described previously, and determine chemical and physical characteristics.

The UV absorbance of A had maxima around 290 and 225 nm, and the solution reacted with sodium nitrite in acid medium. The HPLC peak slightly preceded that of reserpine (see Figure 2). The TLC spot had about the same R_f as aricine, but could be distinguished from aricine by the color (see Figure 1 and Table 1). From the find-

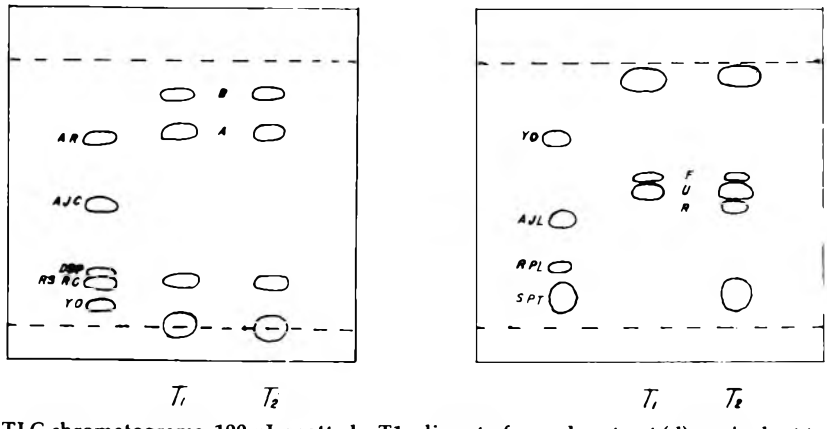

Figure 1. TLC chromatograms, 100 μL spotted. T1, aliquot of sample extract (d) equivalent to 20 mg *Rauwolfia serpentina* concentrated to 1 mL. T2, sample equivalent to 20 mg *Rauwolfia serpentina* extracted with warm methanol; solution was concentrated to 1 mL. At right: developing solvent $CHCl_3$-CH_3OH (80 + 20). At left: developing solvent $CHCl_3$-CH_3OH (97 + 3).

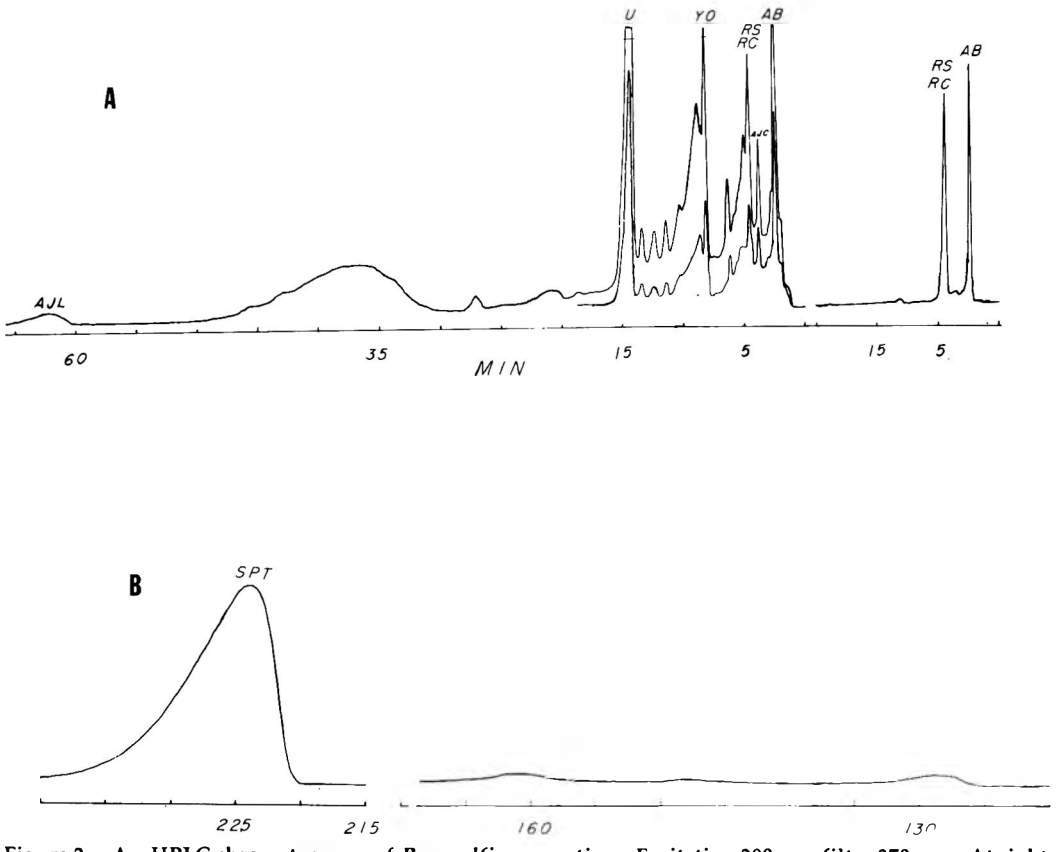

Figure 2. A: HPLC chromatograms of *Rauwolfia serpentina*. Excitation 280 nm, filter 370 nm. At right: 50 μL sample extract (d). At left: about 100 mg *Rauwolfia serpentina* extracted with 50 mL warm methanol; 40 μL injected (top portion); 15 μL injected (lower portion). B: Continuation of top portion of left side.

ings and from information obtained in the literature (5, 14, 15) Compound A was identified as raubasinine (reserpinine). Compound B was isolated and purified, and its physical and chemical characteristics were determined as for A. The UV absorbance curve for B had a peak around 200 nm, but the solution did not react with sodium nitrite in acid medium. The HPLC peak had about the same retention distance as that of A and merged with it (see Figure 2). The TLC spot, however, was higher than that of A and fluoresced blue, whereas A was green. Compound B could not be identified. The UV absorbance curve for Compound U had a peak around 270 nm, and the solution reacted with sodium nitrite in acid medium. In TLC, Compound U came after yohimbine and shortly before ajmaline but it could be distinguished by the color. Compound U could not be identified.

Table 1. Fluorescent colors of TLC spots of *Rauwolfia* alkaloids

Compound or spot	Detection mode[a]	Fluorescent color
B	I	blue
A-raubasinine-reserpinine	I	green
AR	I	orange
AJC	II	dark blue
DSP	II	dark blue
RS; RC	I	green
YO	II	dark blue
F	I	blue
U	I	green
R	I	green
AJL	II	dark blue
RPL	I	yellow
SPT	I	blue

[a] I: longwave UV light; II: shortwave UV light on HF254 plates.

Results and Discussion

Analysis of sample extracts by TLC and HPLC indicated the presence of reserpine, rescinnamine, raubasinine, and unknown compound B. Several other alkaloids, present in the original

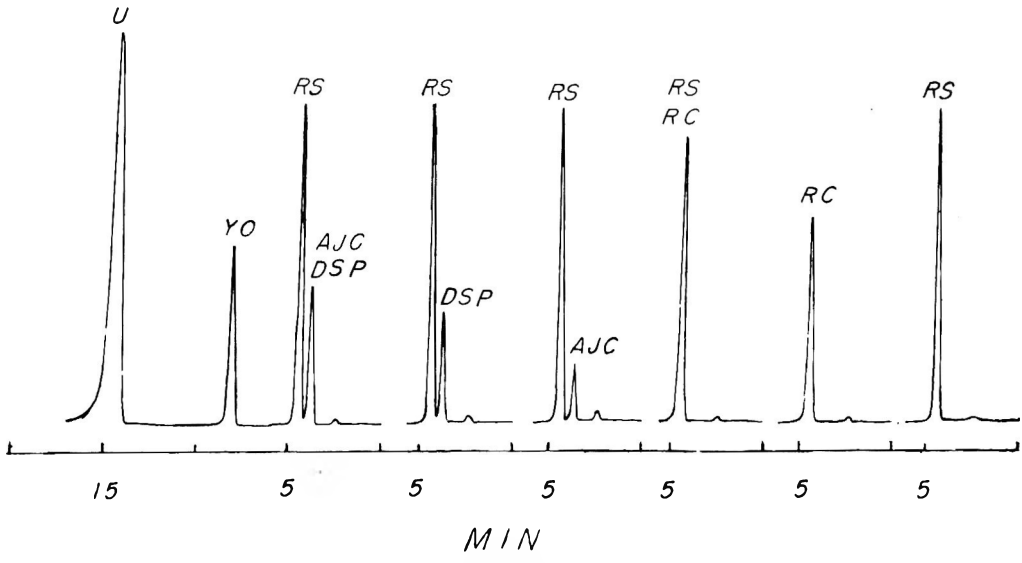

Figure 3. HPLC chromatograms of *Rauwolfia* alkaloids. Excitation 280 nm, filter 370 nm, 50 μL injected. From right to left: RS 0.25 mg/100 mL; RC 0.30 mg/100 mL; RS 0.020 mg and RC 0.06 mg/100 mL; AJC 0.052 mg/100 mL; DSP 0.045 mg/100 mL; AJC 0.052 mg and DSP 0.045 mg/100 mL; U unknown amount.

methanol solution such as ajmalicine, yohimbine, ajmaline, unknown compound U, and serpentine, are retained by the 0.5N acid solution and not extracted by $CHCl_3$. A study was also made of the effect of the silica gel cleanup (12). The column eluates were examined by TLC and HPLC. Results showed that the $CHCl_3$ eluate contained almost exclusively minute amounts of compound B and that the subsequent $CHCl_3$–CH_3OH eluate still contained some of this compound in addition to raubasinine, reserpine, and rescinnamine. As previously indicated, compound B does not react like reserpine, but the presence of raubasinine in the sample extract increases the apparent reserpine-rescinnamine content determined by the USP (2) or AOAC (12) method.

For HPLC, a μPorasil column was used and methanol was the eluting solvent. Reserpine and rescinnamine could not be separated, and formed a single peak with retention time of 4–5 min (Figures 2–4).

Compound B and raubasinine also overlapped and formed a single peak with retention time of about 2 min. With a reverse phase column and also with methanol as solvent, the order of appearance of the peaks is the same, but separation is less satisfactory.

The sample extracts and the methanolic solutions were also examined by TLC for identification. Some separations could be obtained by TLC that could not be achieved by HPLC (see Figures 1–2); for example, Compound B and raubasinine are separated with a developing solvent of $CHCl_3$–CH_3OH (97 + 3) whereas their peaks merge in HPLC. Under the same conditions ajmalicine and deserpidine are separated, but reserpine and rescinnamine form a single

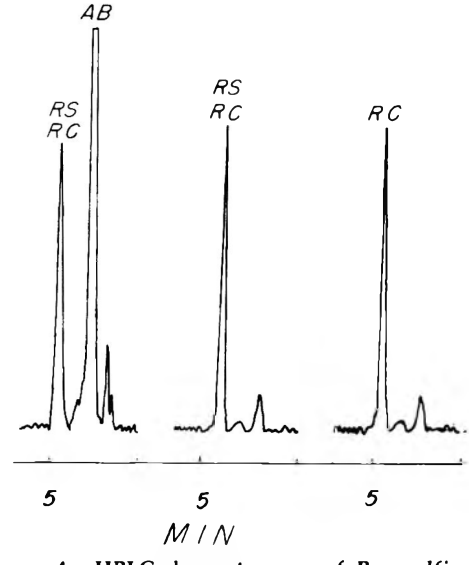

Figure 4. HPLC chromatograms of *Rauwolfia* alkaloids and of *Rauwolfia serpentina*. Excitations 330 nm, filter 370 nm, 50 μL injected. From right to left: RC 0.06 mg/100 mL; RC 0.06 mg and RS 0.20 mg/100 mL. 50 μL sample extract (d).

Table 2. Semiquantitative estimation of serpentine [a]

Sample	SPT, %
Rauwolfia serpentina NF	0.25
Tablets 100 mg	0.18
Tablets 50 mg	0.19

[a] Results indicate percent of powder or of labeled amount of *Rauwolfia serpentina* in tablets.

spot. With a developing solvent of $CHCl_3$–CH_3OH (80 + 20), separations are obtained for yohimbine, Compound U, ajmaline, reserpiline, and serpentine. Compound U is preceded by the spot designated as F and followed by the spot designated as R in Figure 1; the chromatogram contains several additional spots. The spots that fluoresce under longwave UV light, such as those due to raubasinine, Compound B, Compound U, reserpine, and serpentine can be seen at very low levels. Ajmalicine, deserpidine, yohimbine, and ajmaline are detected under shortwave UV light on a green background; these spots are difficult to see at low levels and were not detected in the samples tested.

A semiquantitative TLC procedure was developed for serpentine, the content of which in *Rauwolfia serpentina* samples, as reported in Table 2, was between 0.2 and 0.25%. With the HPLC conditions described here, serpentine is eluted after about 4 h (Figure 2B).

Among compounds that could not be identified, the one designated as U produced the biggest HPLC peak. Since the amount seems considerable, it is hoped that this compound can be identified in the near future. Quantitative determinations of reserpine and rescinnamine by HPLC may be feasible but additional development work is needed. Examination of chromatograms of USP *Rauwolfia serpentina* indicated an approximate reserpine content of 0.1% and an approximate rescinnamine content of 0.04%.

Recommendation

It is recommended that the investigation be continued for the development of a quantitative HPLC procedure for determining reserpine and rescinnamine in *Rauwolfia serpentina*.

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Sources of equipment and reagents are given for convenience of the reader. Equivalent products may also be used.

DRUG RESIDUES IN ANIMAL TISSUES

High Pressure Liquid Chromatographic Determination of Nitrofurazone and Furazolidone in Chicken and Pork Tissues

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A high pressure liquid chromatographic (HPLC) procedure is presented for the determination of nitrofurazone and furazolidone in chicken and pork tissues in the 2-40 ppb range. Muscle, liver, and kidney are homogenized with cold methanol and water (50 + 50). Following methanol evaporation, the nitrofurans are partitioned into ethyl acetate and cleaned up on an alumina column. After elution with 20% methanol in ethyl acetate and evaporation to dryness, residues are determined by HPLC, using a reverse phase analytical column. Overall average recoveries for nitrofurazone and furazolidone were 65.7 and 73.5%, respectively. Average relative standard deviations of 11.9% (nitrofurazone) and 9.5% (furazolidone) at the 2 ppb level were achieved.

In view of the known toxicity of nitrofurans (1), it is important to determine residues of these drugs at the low part per billion (ppb) level in animal tissues.

In Canada, furazolidone is permitted at 55–110 ppm in feed for chickens to prevent hexamitiasis, paracolon infection, typhoid, paratyphoid, and coccidiosis. Furazolidone and nitrofurazone are permitted in swine feed at 330 and 550 ppm, respectively, for the prevention of necrotic enteritis. Nitrofurazone is also used quite extensively as a water-soluble powder for the treatment of caecal coccidiosis in chickens and necrotic enteritis in swine.

Several methods for the quantitative determination of nitrofuran drug residues in animal tissues have been described in the literature. The colorimetric (2) and gas-liquid chromatographic (3, 4) procedures lacked specificity because the nitrofurans were hydrolyzed to a common intermediate, 5-nitrofuraldehyde. A thin layer chromatographic procedure involves conversion to a fluorescent derivative (5).

Because of the transient nature of the metabolites (6, 7) and the structural diversity of the nitrofurans, these drugs should be determined as the intact parent molecules.

High pressure liquid chromatography (HPLC) looks most promising in view of its simplicity and ability to determine nitrofurans without derivatization or other chemical modification. HPLC methods have been reported for furazolidone in poultry tissues (8–10) and for nitrofurazone in milk (11). However, none of these methods has reported simultaneous determination of nitrofurans in animal tissues at a 2 ppb level. In this paper a HPLC method is reported, which is capable of detecting as little as 2 ppb nitrofurazone or furazolidone in chicken and pork tissues.

Experimental

Apparatus

(Wash all glassware with detergent (Decon 75, BDH Chemicals, Toronto, Ontario, Canada M8Z 1K5) and rinse with water, distilled water, and methanol. Let drain and dry.)

(a) *Blender.*—Waring, commercial, with 1 qt jar (Fisher Scientific Co.), or equivalent.

(b) *Mixer.*—Sorvall omni-mixer with 400 mL stainless steel flask (remove gasket) (DuPont Co., Instrument Products, Newtown, CT 06470).

(c) *Syringes.*—Hamilton microliter, blunted needle, 22 gage throughout, 2 in. length (Chromatographic Specialties Ltd, Brockville, Ontario, Canada K6V 5W1).

(d) *Filter funnel.*—350 mL with medium fritted disks (Fisher Scientific Co.).

(e) *Flasks.*—Kimax or Pyrex 500 mL Erlenmeyer flasks, heavy wall for connection to filter vacuum (Fisher Scientific Co.).

(f) *Boiling flasks.*—Kimax or Pyrex 50 or 100 mL pear-shape with ⅋ 15/20 joints, Kimax or Pyrex 500 or 1000 mL round-bottom boiling flasks with ⅋ 24/40 joints (Fisher Scientific Co.).

(g) *Evaporator.*—Buchi/Brinkmann rotary evaporator R with ⅋ 24/40 long stem duct and water bath (Brinkmann Instruments (Canada)

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Ltd, Rexdale, Toronto, Ontario, Canada M9W 4Y5).

(h) *Centrifuge bottles.*—Heavy duty 250 mL glass with ⅋ 27 joint polyethylene stoppers (Kimble 28160) (CanLab.).

(i) *Separatory funnel.*—Pear-shape with ⅋ 22 joint polyethylene stoppers and Teflon stopcocks (Fisher Scientific Co.).

(j) *Centrifuge.*—International PR-J with 384 head for four 250 mL buckets, (Fisher Scientific Co.), or equivalent.

(k) *Funnels.*—60° glass funnel with 100 mm top and 150 mm stem (Fisher Scientific Co.).

(l) *Chromatographic columns.*—No. K-410100 Chromaflex, 6 mm × 200 mm with 50 mL reservoir volume (Kontes Glass Co., Vineland, NJ 08360).

(m) *Liquid chromatograph.*—Model ALC/GPC equipped with U6K injector, Model 440 detector at 365 nm, and M6000A solvent delivery system (Waters Associates Inc., Milford, MA 01757); or Beckman 330 isocratic system with 210 injector, 153 UV detector at 365 nm and 110A solvent delivery system (Beckman Instruments Inc., Berkeley, CA 94710), or equivalent (isocratic function only required).

(n) *Column.*—Brownlee RP-10A, reverse phase, C-8 (RP-8), 10 μm 4.6 mm id × 25 cm. Use Brownlee RP-GU HPLC guard columns with C-8 (RP-8) 10 μm packing for liver and kidney samples (Technical Marketing Associates Ltd, 1661 Finfar Court, Mississauga, Ontario, Canada L57 4K1). Operating conditions: mobile phase acetonitrile–water (40 + 60 for muscle, 30 + 70 for liver and kidney) made 0.01M with respect to ammonium acetate, flow rate 1.5 mL/min; fixed wavelength UV detection at 365 nm.

Reagents

(a) *Solvents.*—Dimethylformamide, methanol, and ethyl acetate, distilled in glass grade; acetonitrile, HPLC grade with 190 nm UV cut-off (Caledon Laboratories, Georgetown, Ontario, Canada L7G 4R9). Water, deionized through Millipore Milli-Q system (Millipore Corp., Bedford, MA 01730).

(b) *Extracting solvent.*—Methanol–water (50 + 50) for muscle. Chill to 0–4°C. Include 0.2% metaphosphoric acid in this solvent for liver and kidney after initial dissolution in the water portion.

(c) *Mobile phase.*—Acetonitrile–water (40 + 60 for muscle) (30 + 70 for liver and kidney) made 0.01M with respect to ammonium acetate. Prepare by diluting 100 mL 0.1M ammonium acetate with 400 or 300 mL acetonitrile and 500 or 600 mL water to 1 L. Degas before use.

(d) *Alumina.*—Neutral, Brockmann Activity No. 1, 80–100 mesh, Fisher A-950.

(e) *Standard solutions.*—(Nitrofurazone and furazolidone were donated by Norwich-Eaton Pharmaceuticals, Norwich, NY 13815.) Store stock and working solutions in low actinic volumetric flasks. (*1*) *Stock solution.*—1 mg/mL. Dissolve 50 mg of each nitrofuran in dimethylformamide and dilute to 50 mL. (*2*) *Working solution.*—1 ng/μL (serves as fortification standard for recovery studies and HPLC standard). Dilute 1 mL stock solution to 100 mL with mobile phase and dilute 1 mL of this dilution to 10 mL with mobile phase (i.e., a 1:1000 composite dilution). Prepare working solution fresh daily. Protect standard solutions from sunlight and fluorescent light. Wrap 10 mL flasks in aluminum foil.

(f) *Other standards.*—Nihydrazone was also donated by Norwich-Eaton; nifursol was provided by Salsbury Laboratories, Charles City, IA 50616. Nitrofurantoin was purchased from Sigma Chemical Co., St. Louis, MO 63178.

Preparation of Tissues for Recovery Experiments

Pool mixed breast and thigh muscle from white leghorn hens (1.5 kg) and blend in Waring blender. Weigh 10 g portions to nearest 0.1 g and store frozen at −20°C wrapped in aluminum foil. Similarly process chicken liver and pork back muscle, liver, and kidney.

Procedure

Modify laboratory lighting to obtain alternative incandescent lighting, preferably with dimmers. Approximately 600 watts/900 sq. ft is recommended. Ceiling bulbs or bench lamps may be used. All fluorescent lighting and sunlight must be blocked from laboratory while work is in progress. Black out windows with cardboard or other material.

Extraction from muscle.—Place 10 g tissue into a 400 mL stainless steel flask. Fortify with standard onto surface of tissue and immediately add 100 mL cold extracting solvent. Homogenize 2 min at medium speed and 10 s at high speed. Rinse blades. Filter through 10 g Celite 545 on medium fritted disk funnels after prewashing the assembly with 100 mL cold extracting solvent. (Use water aspirators for filtration and roto-evaporation.) Rinse flask with 100 mL extracting solvent and transfer rinse to funnels. Transfer pooled filtrates from 500 mL vacuum flasks to 1000 mL boiling flasks with three 10 mL wash bottle rinsings of extracting

solvent. Roto-evaporate to <50 mL at 40–45°C (45 min) and transfer, with water rinsings, to 250 mL separatory funnels containing 10 g sodium chloride. Dissolve salt and dilute to ca 80 mL with water. Add 20 mL chilled 1N HCl. Let stand 5 min and partition into two 100 mL portions of ethyl acetate with 5 min shaking periods of ca 1 shake/s. Transfer ethyl acetate to 500 mL Erlenmeyer flask containing 15 g anhydrous sodium sulfate and 2 g sodium carbonate to dry and neutralize traces of hydrochloric acid. Swirl for a few minutes. Decant through 100 mm funnel containing a glass wool plug (prewash with ethyl acetate) into 500 mL boiling flask with ethyl acetate rinsings and evaporate to dryness. At this point extracts can be stored overnight in freezer.

Extraction from liver and kidney.—Except for the following modifications, the procedure is identical to that for muscle. Include 0.2% metaphosphoric acid in extracting solvent (see *Reagents*). It may be preferable to use 250 mL glass centrifuge bottles for partitioning rather than separatory funnels so that emulsions can be broken (centrifuge at 2000 × *g* for 5 min).

Pour 2 g alumina into glass wool-plugged Chromaflex columns (wash plugged columns with methanol and let dry overnight). Place 100 mL pear-shape flasks under columns. Reconstitute residues in 3 mL ethyl acetate and transfer to column. Rinse flask with another 3 mL ethyl acetate and transfer to column. Elute nitrofurans with 20 mL of 20% methanol in ethyl acetate. Collect all eluant and roto-evaporate to dryness. Dissolve residue in 500 μL mobile phase and inject a 200 μL aliquot into the HPLC system. Use guard columns for liver and kidney samples (see *Apparatus*).

High pressure liquid chromatography.—Prepare standard curves for nitrofurazone and furazolidone by plotting peak heights vs concentrations for 200 μL injections of extracts from tissues fortified with 20, 100, 200, 300, and 400 μL working solution (2, 10, 20, 30, and 40 ppb). Recovery for each concentration was determined by comparing these peak heights to those for direct HPLC injections of 8, 40, 80, 120, and 160 μL working solution (2, 10, 20, 30, 40 ppb), respectively.

Results and Discussion

Of several extracting solvents for tissue analysis of nitrofurazone and furazolidone, a cold methanol–water (50 + 50) mixture gave the least interference, and had the capability of volume reduction by evaporation. Although metaphosphoric acid is not required for muscle extraction, it is mandatory for liver or kidney extraction to precipitate soluble material which would otherwise clog the Celite.

Centrifuge bottles and separatory funnels are used interchangeably for ethyl acetate partitioning of liver and kidney extracts. The advantage of the centrifuge bottle is that the occasional persistent emulsion may be removed by centrifugation. On the other hand, with a separatory funnel, the interphase may be displaced to the constriction after the second extraction, and small ethyl acetate rinsings more easily collected.

The use of small alumina columns for extract cleanup is mandatory to allow quantitation of nitrofurans at 2 ppb levels in tissues. Without the column cleanup, much polar material would have remained, obscuring the peaks, particularly nitrofurazone. Each batch of alumina should be calibrated and the elution of nitrofuran standards verified before use. One should observe recoveries of approximately 75 and 90% of 100 ng nitrofurazone and furazolidone, respectively. Increased polarity of the eluant did not improve the recovery of nitrofurazone.

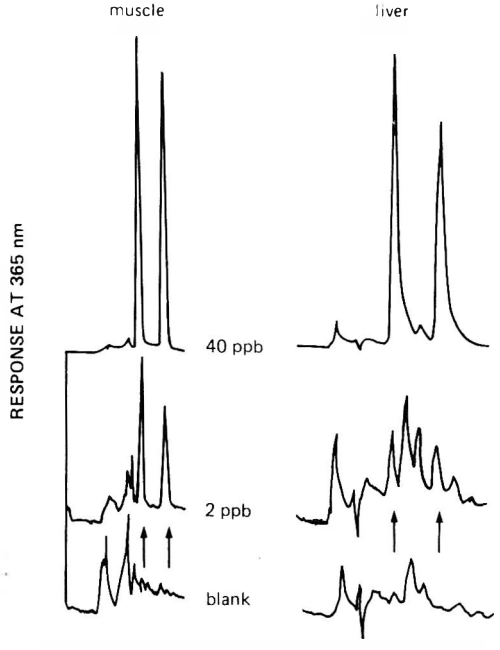

Figure 1. Chromatograms of chicken tissues, blanks and fortified at 2 and 40 ppb. Mobile phase was acetonitrile–water (40 + 60 muscle; 30 + 70, liver) made 0.01M with respect to ammonium acetate. Attenuation 0.005 AUFS for blanks and 2 ppb, 0.04 AUFS for 40 ppb.

Table 1. Standard curve and recovery data for nitrofurazone in chicken and pork tissues

Added ng/g (ppb)	Peak ht,[a] mm	Std dev.	Rel. std dev., %	Rec., %
		Chicken Muscle		
2	29.6	2.6	8.8	76.0
10	120.3	10.5	8.7	66.5
20	242.0	27.1	11.2	69.2
30	387.3	27.3	7.1	74.6
40	496.7	55.5	11.2	73.7
	$y = 12.5x - 0.8$; $r = 0.985$			
		Pork Muscle		
2	20.3	3.1	15.3	60.0
10	82.5	5.7	6.9	54.0
20	159.0	18.8	11.8	54.0
30	240.0	26.8	11.2	56.0
40	352.0	21.6	6.1	61.0
	$y = 8.6x - 3.98$; $r = 0.996$			
		Chicken Liver		
2	15.6	1.8	11.8	71.9
10	66.4	6.4	9.7	68.1
20	140.8	15.1	10.7	61.9
30	218.6	23.8	10.9	63.9
40	298.8	34.4	11.5	66.6
	$y = 7.4x - 1.0$; $r = 0.982$			
		Pork Liver		
2	17.7	1.5	8.4	73.1
10	71.4	3.1	4.3	63.1
20	136.4	4.4	3.2	66.2
30	223.2	1.4	0.6	70.2
40	292.0	1.8	0.6	71.4
	$y = 7.3x - 0.9$; $r = 0.999$			
		Pork Kidney		
2	14.5	2.2	15.1	62.9
10	65.8	2.6	4.0	59.2
20	120.7	2.7	2.2	58.0
30	210.0	1.0	0.5	68.2
40	292.7	2.2	0.8	73.9
	$y = 7.3x - 8.4$; $r = 0.995$			

[a] Uses 2 dilutions of each of 3 stock solutions to obtain 6 standard curves and estimates of recovery.

Typical chromatographic tracings for chicken tissues and pork tissues are shown in Figures 1 and 2, respectively. As in the case of other studies (2, 8, 9, 12), this work did not include chicken kidney which is normally not consumed by humans and for which metabolic studies have revealed lower levels than for liver (3, 12–14). There is very little interference in muscle extracts at 2 ppb and although more occurs in liver extracts (as well as kidney), the resolution is sufficient to allow quantitation with a coefficient of variation not exceeding 20% (see Tables 1 and 2) at this level.

Standard curves and recoveries were established on fortified tissues for both chickens and swine. The data are shown in Tables 1 and 2. The linearity of the data with little scattering is evident from the high correlation coefficients observed from linear regression analyses of peak height vs concentration for 6 runs. For a 2–40 ppb concentration range, an acceptable analysis should have an interlaboratory variation not exceeding 30%, which translates to approximately 15–20% within-laboratory variation (15). It is evident that all data in Tables 1 and 2 more than meet this criterion. At 2–40 ppb, recoveries

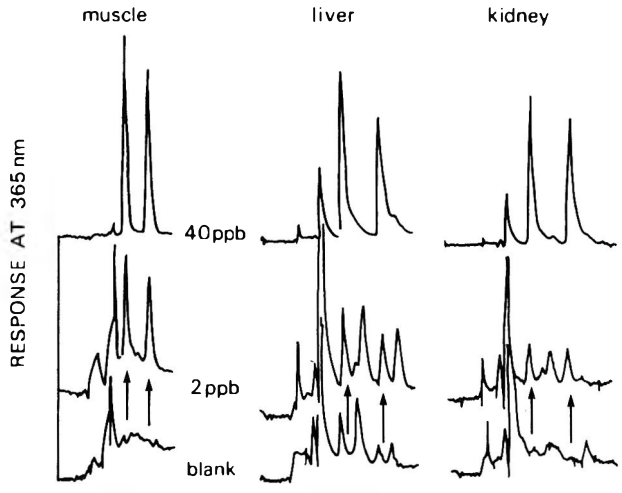

Figure 2. Chromatograms of pork tissues, blanks and fortified at 2 and 40 ppb. See Figure 1 for other details. Conditions for kidney same as for liver.

of more than 60% are sought. Overall recovery in this procedure exceeds this figure in spite of some lower values. For nitrofurazone and furazolidone, average recoveries were 65.7 and 73.5%, respectively. Loss occurs on the alumina column, but this tends to be fairly constant, which perhaps accounts for the maintenance of precision. Nitrofurazone and furazolidone showed average relative standard deviations of 11.9 and 9.5%, respectively, at 2 ppb. It is this precision which permits the usefulness of the procedure, because recovery is accounted for in the calculation of results.

Another potentially important loss is through the action of light. The exclusion of all sunlight and fluorescent lighting (even from corridors) is absolutely essential. Even a small loss of approximately 1%/h can occur with incandescent lighting. In view of these measures, low actinic glassware was not employed other than for standards and because it was desired to visually monitor cleanliness and operations. Finally, some loss may occur through protein binding.

The use of Decon 75 for washing glassware was considered simpler and safer than acid washing and subsequent silylation (5, 10) and does not introduce extraneous peaks (10). Differences in furazolidone cleanup between the present method and others (10) could be ascribed to other factors besides silylation, such as column cleanup.

Retention times for nitrofurazone and furazolidone are shown in Table 3 for acetonitrile–water mobile phases used for muscle (40 + 60) and liver and kidney (30 + 70). Use of the latter mobile phase slows analysis time but allows optimum resolution from tissue interferences. The data also indicate good resolution from other nitrofurans. Nifursol will not separate from nitrofurantoin but the latter is not used in veterinary practice. The whole procedure has not been extended to all these nitrofurans although some initial extractions with nihydrazone show promise for this nitrofuran.

To validate the method, a trial was undertaken to determine residues in dosed animals. Four white leghorn hens of approximately 1.5 kg were dosed with 50 mg each of nitrofurazone and furazolidone after being deprived of feed for 24 h. Two hens were sacrificed 8 h later and two 24 h after administration. Another 2 hens were maintained on the same feed to serve as controls. Liver and thigh muscle were quickly chilled, frozen, and analyzed over a 2 week period. The results in Table 4 depict a much higher level of nitrofurazone than furazolidone in the tissues. The absence of detectable furazolidone or the presence of very low levels after 8 to 24 h is not surprising. Previous findings showed a low plateau in chicken liver after 8 h (13) and a complete absence after 24 h (12). This corresponds to the work by Ritchie et al. (3) although the inverse had been shown with nitrofurazone (14).

It should be noted that thigh and breast chicken muscle can be used interchangeably since extraction of fortified thigh muscle was checked and found to be the same as that for mixed breast and thigh muscle.

With this procedure it is possible for one ana-

Table 2. Standard curve and recovery data for furazolidone in chicken and pig tissues

Added ng/g (ppb)	Peak ht,[a] mm	Std dev.	Rel. std dev., %	Rec., %
		Chicken Muscle		
2	26.2	2.0	7.7	86.3
10	122.0	10.2	8.4	80.3
20	241.0	28.7	11.9	81.8
30	356.8	35.5	10.0	82.4
40	469.0	59.0	12.6	80.2
	$y = 11.7x + 4.7$; $r = 0.984$			
		Pork Muscle		
2	15.7	2.0	12.7	67.0
10	67.3	7.6	11.3	60.0
20	126.7	17.1	13.5	59.0
30	182.0	17.1	9.4	52.0
40	280	15.6	5.6	66.0
	$y = 6.7x - 2.57$; $r = 0.994$			
		Chicken Liver		
2	15.2	1.0	6.5	88.1
10	69.0	2.4	3.5	78.3
20	131.4	10.4	7.9	74.0
30	207.4	16.5	8.0	79.0
40	285.0	27.9	9.8	78.7
	$y = 6.9x - 1.1$; $r = 0.987$			
		Pork Liver		
2	10.2	0.94	9.2	62.6
10	49.6	2.90	5.8	63.4
20	97.5	3.60	3.7	67.1
30	165.3	0.78	0.5	73.1
40	218.5	1.31	0.6	76.1
	$y = 5.56x - 5.19$; $r = 0.998$			
		Pork Kidney		
2	11.4	1.31	11.5	73.2
10	56.2	1.50	2.7	74.6
20	102.7	1.37	1.3	71.7
30	169.3	0.83	0.5	78.2
40	233.3	1.80	0.8	84.9
	$y = 5.81x - 3.92$; $r = 0.998$			

[a] Uses 2 dilutions of each of 3 stock solutions to obtain 6 standard curves and estimates of recovery.

lyst to complete the extraction and cleanup of 6 tissues in a working day. Beyond this, efficiency depends on such factors as wash-up support and equipment such as supplementary roto-evaporators and automatic sampling for HPLC.

The major result of this study was to show that

Table 3. Retention time (R_t) of nitrofuran drugs on RP-8 column with 2 acetonitrile–water mobile phases

	40 + 60 (Muscle)		30 + 70 (Liver and kidney)	
Nitrofuran	R_t (min)	Rel. R_t	R_t (min)	Rel. R_t
Nitrofurazone	2.75	1.00	3.91	1.00
Furazolidone	3.73	1.35	5.71	1.46
Nifursol	2.62	0.95	—	—
Nitrofurantoin	2.63	0.95	—	—
Nihydrazone	3.04	1.10	—	—

Table 4. Determination of nitrofurazone and furazolidone in hens dosed with 50 mg of each drug

Animal No.	Time, h[a]	Nitrofurazone, ppb	Furazolidone, ppb
		Muscle	
1	8	534	10
2	8	1207	20
3	24	8	ND[b]
4	24	6	ND
		Liver	
1	8	50	ND
2	8	68	ND
3	24	6	ND
4	24	5	ND

[a] Time from dosage to sacrifice.
[b] ND = not detected.

both nitrofurazone and furazolidone can be determined quantitatively in chicken and pork tissues at 2–40 ppb. Previous studies had determined furazolidone in chicken tissues at 10 ppb (8) and at 2 ppb (9) and 0.5 ppb (10) in turkey tissues, but the present technique extends this capability to nitrofurazone and to quantitation of both drugs at 2 ppb.

Acknowledgment

The authors acknowledge the skilled technical assistance of G. H. Blondin.

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Quantitative Thin Layer Chromatographic Multi-Sulfonamide Screening Procedure

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In-situ optical scanning of fluorescamine derivatives on thin layer silica gel plates provides a rapid method for the determination of multiple sulfonamides at levels below 0.1 ppm. Sample preparation is minimal. Homogenized liver or muscle is extracted with ethyl acetate and then back-extracted into 0.2M glycine buffer. After pH adjustment, the extract is washed with hexane and extracted with methylene chloride. The organic phase is evaporated to dryness and reconstituted in methanol. Pre-adsorbent layer silica gel plates are used for chromatography. The method has been applied to residues of sulfamethazine, sulfadimethoxine, sulfathiazole, sulfaquinoxaline, and sulfabromomethazine in cattle, swine, turkey, and duck tissues.

To overcome the problem of poor specificity inherent in spectrophotometric determinations of sulfonamide residues, such as the method of Tishler (1), a number of chromatographic procedures have been proposed. A recent review (2) of sulfonamide methodology cites gas chromatography (GC) (3–5), high performance liquid chromatography (HPLC) (6), and gas chromatography/mass spectrometry (7, 8) as approaches capable of reliable sub-parts per million level quantitation for sulfonamide residues in tissue. Although one thin layer chromatographic (TLC) approach (9) is cited, the technique is described by the reviewer as lacking the necessary precision for quantitation. In fact, TLC has several distinct advantages (9–13) over other chromatographic techniques for rapid analysis of trace components. The capacity to analyze many samples simultaneously rather than serially results in significant time savings, a primary consideration in the analysis of large numbers of samples. Furthermore, when a multi-component mixture is to be separated, solvent systems can be chosen without regard for potential incompatibility with the detection mechanism which, for TLC, unlike GC or HPLC, is off-line. This allows optimization of detector sensitivity and selectivity independent of chromatographic resolution. In addition, the speed of analysis using an automated TLC scanner (about 1 sample per minute) reduces equipment needs because a single instrument can perform different assays without elaborate reconfiguration (i.e., no column changes, pump equilibration).

Nanogram quantities of sulfonamides can be detected on TLC plates with either the Bratton–Marshall reagent (3) or fluorescamine (9–13). The latter has inherent advantages in terms of linearity of response and baseline stability for densitometry. The selectivity afforded by fluorescence determination allows the use of simpler cleanup techniques.

This paper presents the extension of our earlier work (12, 13) to a variety of sulfonamides and animal species. The accuracy and repeatability of the method near the official tolerance, 0.1 ppm (14), are demonstrated. The use of an internal standard and pre-adsorbent spotting zones provides significant improvements in both accuracy and precision. The results of a collaborative

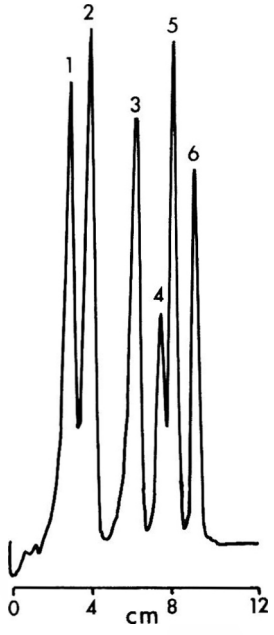

Figure 1. Separations of 6 sulfonamides, including internal standard: 1, sulfathiazole; 2, sulfapyridine, internal standard; 3, sulfamethazine; 4, sulfaquinoxaline; 5, sulfadimethoxine; 6, sulfabromomethazine. Approximately 100 ng of each component.

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Table 1. Determination of sulfonamides (ppm) in fortified tissues [a]

	Beef		Turkey		Duck	
Added	Liver	Muscle	Liver	Muscle	Liver	Muscle
			SMZ			
0.05	0.053	0.051	0.053	0.051	0.052	0.048
CV	4.3	2.2	5.5	5.5	2.9	3.7
0.10	0.103	0.102	0.102	0.104	0.104	0.100
CV	6.0	3.4	2.2	3.1	3.9	2.7
0.20	0.192	0.197	0.198	0.197	0.199	0.195
CV	4.6	4.8	1.7	2.0	3.6	3.5
			SDM			
0.05	0.053	0.050	0.052	0.053	0.054	0.057
CV	5.1	3.0	5.9	5.7	2.7	9.3
0.10	0.104	0.104	0.103	0.105	0.108	0.101
CV	5.3	8.0	3.1	4.1	1.5	3.8
0.20	0.194	0.197	0.191	0.196	0.196	0.196
CV	6.8	11.2	3.2	3.1	3.2	4.0
			SQX			
0.05			0.049	0.052	0.049	0.052
CV			6.0	6.5	5.3	6.5
0.10			0.097	0.105	0.106	0.104
CV			5.6	3.0	6.7	3.6
0.20			0.190	0.200	0.200	0.207
CV			5.4	4.0	7.7	3.0
			STZ in Swine		SBR in Beef	
0.05			0.051	0.050	0.052	0.050
CV			10.0	7.4	3.7	10.0
0.10			0.094	0.101	0.098	0.105
CV			10.0	4.9	3.2	8.0
0.20			0.209	0.199	0.195	0.197
CV			7.7	4.8	5.3	3.9

[a] Six replicates at each level

study of the procedure will be reported in another paper (15).

Results and Discussion

A comparison of the results of analyses of single sulfonamides (Table 1) with those of simultaneous multiple determinations (Table 2) indicates no loss of accuracy and only a small increase in the coefficient of variation (Δ CV = 0.4–1.7%). Therefore, the chromatographic resolution (Figure 1) is sufficient for accurate multi-residue determinations. The data also suggest slightly more variability in the analysis of red meat (composite CV = 5.6%) compared with poultry (composite CV = 4.2%). This is readily understandable in terms of the relative fat content of the different species.

In our initial work, an acidic extraction of the ethyl acetate phase was used to minimize fatty acid carryover. Unlike sulfamethazine, many of the sulfonamides have unfavorable acid–ethyl acetate partition ratios which necessitate an alkaline extraction. When 0.1M NaOH was used for this purpose, the pH of the aqueous phase decreased to the 8–9 range after ethyl acetate partition, too low for adequate recovery of several sulfonamides. Glycine buffer, pH 12.25, had sufficient buffering capacity to maintain the pH greater than 10, greatly improving recoveries. Washing the aqueous phase at pH 5.25 with hexane reduces the amount of fat in the final extract. Even with this step, a residue of oil that is insoluble in methanol remains after evaporation of the methylene chloride to dryness. Irregular chromatography and poor quantitation resulted whenever this liquid phase was co-spotted with the methanol fraction. Because sampling the methanol layer without disturbing the oily residue is not difficult and a rapid procedure was desired, no attempts were made at further cleanup of the samples.

Overall results for SMZ, SDM, SQX, STZ, and

Table 2. Simultaneous determination of SMZ, SDM, and SQX in fortified swine tissues at 0.1 ppm

Tissue	Compound	Mean ppm[a] found	Mean within-day CV ($n = 4$)	Day-to-day CV
Liver	SMZ	0.101	4.7	3.3
	SDM	0.101	5.8	3.7
	SQX	0.101	5.6	7.3
Muscle	SMZ	0.103	3.9	1.4
	SDM	0.103	6.3	2.8
	SQX	0.104	4.1	1.7

[a] Twelve replicates per day for 4 days.

SBR are comparable in terms of accuracy but the composite CVs demonstrate a trend in variability; SMZ (3.8%) < SDM, SQX (5.2%) < SBR (5.7%) < STZ (7.5%). This correlates reasonably well with the shape of their respective partition curves between methylene chloride and aqueous phosphate buffer solutions of varying pH and with the fact that the partition characteristics of the internal standard, SPY, are closest to SMZ.

The accuracy and precision of this approach is superior to a recently collaboratively studied GC procedure (16) and comparable to the much more sophisticated GC-MS technique (8, 16). Combined with high sample capacity (16–24 samples per day), this level of performance makes quantitative TLC an attractive choice for sulfonamide residue monitoring.

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Quantitative Thin Layer Chromatographic Multi-Sulfonamide Screening Procedure: Collaborative Study

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A thin layer chromatographic procedure suitable for detection of multiple sulfonamides at 0.1 ppm was studied in an interlaboratory collaborative study. Sulfamethazine, sulfadimethoxine, and sulfaquinoxaline were variously analyzed in liver and muscle tissues from swine, turkey, and duck. The average recovery for all drugs across all tissues was 95%. The corresponding repeatability and reproducibility were 7.7% and 10.5%, respectively.

Recently, a collaborative study (1) of a gas chromatographic/mass spectrometric (GC/MS) procedure, a gas chromatographic (GC) procedure, and the Tishler colorimetric procedure was conducted. Of the 3 procedures, the GC/MS method was the most accurate and precise by a substantial margin. Recognizing concern for the high cost of GC/MS instrumentation, the Food Safety and Inspection Service (FSIS) decided to collaboratively study the thin layer chromatographic (TLC) procedure originally proposed (2) as a screen prior to GC/MS analysis. The design of the study was similar to that reported in ref. 1. Blind duplicates of 4 levels of fortified samples and 3 levels of incurred residue samples (at least one pair of blank tissue), each in liver and muscle, were used for each species and drug or drug combination. Species and drugs were chosen on the basis of current violation rates and allowed usage. Samples were analyzed by adding a 100 μL aliquot of drugs and internal standard (for fortified samples) or internal standard alone (for incurred samples) to the appropriate tissue. Samples were analyzed in 8 sets of 17 samples (8 fortified, 6 incurred, 3 knowns for standard curve). Except for differentiation of incurred and fortified, samples were blind and the order of analysis was completely randomized. Analysts were requested to report results to 3 decimal places for statistical evaluation. The specific experimental sets are shown in Table 1.

Table 1. Sample set identification

Set	Species	Tissue	Drug(s)
1	turkey	muscle	sulfaquinoxaline
2	turkey	liver	sulfaquinoxaline
3	turkey	muscle	sulfamethazine sulfadimethoxine
4	turkey	liver	sulfamethazine sulfadimethoxine
5	swine	muscle	sulfamethazine sulfadimethoxine
6	swine	liver	sulfamethazine sulfadimethoxine
7	duck	muscle	sulfadimethoxine
8	duck	liver	sulfadimethoxine

Samples

Swine were obtained from the Agricultural Research Service, U.S. Department of Agriculture. The animals were maintained on medicated feed containing Aureo SP-250 at predetermined levels by Lowell Frobish of the Non-Ruminant Nutrition Laboratory, Agricultural Research Center, Beltsville, MD. One group of turkeys was dosed with Rofenaid 40 (supplied by Alexander MacDonald, Hoffmann-La Roche, Inc.), a mixture of sulfadimethoxine and ormetoprim. A second group was placed on a similar diet with a sulfaquinoxaline premix used instead of the Rofenaid 40. The animals were maintained by David Wagner, Bureau of Veterinary Medicine, Food and Drug Administration, Beltsville, MD. Ducks were also fed medicated feed containing Rofenaid 40. These animals were treated and slaughtered by the Long Island Duck Growers Association.

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The recommendation of the Associate Referee was approved by the General Referee and Committee G and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

[1] U.S. Department of Agriculture, Food Safety and Inspection Service, Mathematics and Statistics Division, Washington, DC 20250

Sulfonamides in Animal Tissues

Thin Layer Chromatographic Screening Method

Official First Action

(Applicable to swine, turkey, and duck tissues)

41.D01 *Principle*

Sulfonamides are extd with ethyl acetate after addn of sulfapyridine as internal std. Exts are cleaned up by partitioning between org. and aq. solvs, and chromatographed on silica gel TLC plates. Developed plates are treated with fluorescamine and scanned by fluorescence densitometer.

41.D02 *Reagents*

(a) *Ethyl acetate, hexane, methylene chloride, and methanol.*—Distd in glass (Burdick & Jackson Laboratories, Inc.).

(b) *Glycine buffer soln.*—Prep. glycine (Fisher Scientific Co.) as 0.2M aq. soln and adjust pH to 12.25 with NaOH.

(c) *Fluorescamine derivatizing soln.*—Dissolve 25 mg fluorescamine (Pierce Chemical Co.) in 250 mL acetone. Replace soln after treating 8–9 plates.

(d) *Sulfonamide stds.*—Com. sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfathiazole (STZ), sulfabromomethazine (SBR), and sulfapyridine (SPY).

(e) *Stock std solns.*—Dissolve 100 mg sulfonamide in 100 mL acetone. Store in refrigerator.

(f) *Fortification std solns.*—Combine sulfonamides of interest and dil. to 5.00, 2.50, and 1.25 μg/mL (equiv. to 0.2, 0.1, and 0.05 ppm in tissue), using 0.05M pH 7.5 phosphate buffer. All solns should contain 2.50 μg SPY/mL. Store fortification stds in refrigerator and prep. weekly.

(g) *Internal std soln.*—Using stock std soln (e), prep. 2.50 μg SPY/mL 0.05M pH 7.5 phosphate buffer.

41.D03 *Apparatus*

(a) *Densitometer.*—CAMAG TLC/HPTLC scanner (Applied Analytical Industries, Wilmington, NC 28403) equipped with 400 nm interference filter on excitation source. Replace std 400 nm cutoff filter on photomultiplier by 500 nm interference filter. Slit dimensions 7.8 × 0.3 mm. Scan plates at either 1 or 2 mm/s.

(b) *TLC plates.*—20 × 20 cm channeled plates, channels 8 cm wide with 0.25 mm silica gel layer and pre-adsorbent spotting zones (Whatman LK6D, Whatman, Inc.).

(c) *TLC spotting capillaries.*—20 μL glass capillary tubes (Corning Glass Works).

(d) *Heat strip.*—Automatic spotter heat strip set at 85° (Analytical Instrument Specialties, Inc., Libertyville, IL 60048). Any equiv. heating device with temp. control can be substituted.

(e) *TLC tank.*—Std 2-trough tank (Alltech Associates, Deerfield, IL 60015), atm. fully satd by lining with satn pads.

(f) *Derivatization tank.*—Stainless steel (A. H. Thomas).

(g) *Evaporator.*—N-Evap (Organomation Associates, Inc., Northborough, MA 01532).

(h) *Homogenizer.*—Tekmar SDT Tissumizer (Tekmar Co., Cincinnati, OH 45222).

(i) *Shaker.*—Horizontal reciprocating shaker set at ca 240 cycles/min (Eberbach Corp., Ann Arbor, MI 48106).

(j) *Centrifuge.*—Set at 2500 rpm for 5 min (International Equipment Co., Needham Heights, MA 02194).

(k) *Polypropylene centrifuge tubes.*—50 mL capacity (Corning Glass Works).

41.D04 *Sample Extraction*

Accurately weigh ca 2.5 g homogenized liver or muscle into 50 mL centrf. tube. Add 100 μL internal std soln. Prep. 3 control samples (using tissue known to be free of sulfonamides) and fortify with internal std (0.1 ppm) and each sulfonamide of interest, one control each at 0.05, 0.10, and 0.20 ppm. Wait 15 min and then add 25 mL ethyl acetate. Blend muscle samples 1 min with Tissumizer, and centrf. For liver samples, cap tube tightly, shake 20 min on horizontal shaker, and centrf. Transfer ethyl acetate to clean tube and discard tissue. Add 10 mL glycine buffer to ext, mech. shake 5 min, and centrf. Vac.-aspirate and discard org. phase. Adjust pH of aq. phase to 5.2–5.3 by adding 2 mL (1 + 1) mixt. of 2M pH 5.25 phosphate buffer and 1.7M HCl. Check pH and make final adjustments with either addnl buffer or 0.1N NaOH. Add 5 mL hexane, mech. shake 5 min, and centrf. Aspirate and discard hexane phase. Remove any solid or emulsified material remaining at interface. Add 10 mL CH_2Cl_2, shake 5 min, centrf., and aspirate and discard aq. phase. Add 10 μL diethylamine to CH_2Cl_2 ext and conc. just to dryness under stream of N at 40°. During evapn, occasionally rinse tube walls with CH_2Cl_2. Redissolve residue in 100 μL MeOH and mix 30 s on vortex mixer. Let stand 5 min before chromatgy to let insoluble oils settle.

41.D05 ***Chromatography***

Apply 20 μL portion of sample to pre-adsorbent spotting zone of TLC plate. Avoid using lane at each side of plate, and spot the 3 fortified control samples at intervals across plate to minimize effects of across-plate variation. Develop plate 1 cm in MeOH followed by 2 developments, 6 cm and 12 cm, in $CHCl_3$–*tert*-BuOH (80 + 20). Between each development, dry plate 1 min at 110°. If STZ is suspected or present, prewash $CHCl_3$–*tert*-BuOH with H_2O. Maintain development tank temp. at 30–33° for optimum resolution of multiple sulfonamides. Derivatize compds by quickly dipping plate in fluorescamine soln. Bands are visible after 15–30 min. Scan each lane and obtain its response scanning curve. For each sample and std (each lane), det. ratio of response for sulfonamide of interest to response for internal std (SPY).

41.D06 ***Calculations***

For each sulfonamide, calc. slope and intercept of std curve, concn vs response ratio, using linear regression and results for the 3 fortified samples. Plotting sulfonamide concn on *y*-axis results in std error of est. ($S_{y.x}$) having dimension of ppm, thus simplifying estn of confidence intervals. For quality assurance purposes, $S_{y.x}$ should be $\leq$0.02 ppm, and correlation coefficient, *r*, should be $\geq$0.995. Use linear regression slope and intercept to calc. concn of samples from their respective sulfonamide/internal std peak ht ratios.

Table 2. Collaborative results for sulfaquinoxaline in turkey (ppm)

	Laboratory							
Level	1	2	3	4	5	6	7	8
Fortified				Muscle				
0.06	0.076	0.060	0.072	0.069	0.051	0.069	0.065	0.053
	0.076	0.060	0.072	0.079	0.048	0.074	0.070	0.065
0.11	0.126	0.106	0.100	0.113	0.099	0.109	0.124	0.104
	0.112	0.107	0.101	0.132	0.057	0.102	0.128	0.094
0.14	0.132	0.129	0.112	0.155	0.116	0.131	0.153	0.122
	0.137	0.118	0.129	0.131	0.121	0.114	0.166	0.121
0.18	0.180	0.161	0.142	0.179	0.111	0.150	0.185	0.136
	0.151	0.126	0.133	0.210	0.179	0.144	0.213	0.168
Incurred				Muscle				
1	0.057	0.068	0.065	0.079	0.049	0.067	0.049	0.032
	0.054	0.080	0.070	0.071	0.067	0.082	0.049	0.039
2	0.169	0.158	0.205	0.115	0.159	0.195	0.199	0.160
	0.175	0.148	0.188	0.160	0.134	0.205	0.185	0.158
3	ND[a]	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND
Fortified				Liver				
0.06	0.089	0.077	0.087	0.070	0.061	0.062	0.066	0.063
	0.081	0.057	0.078	0.073	0.056	0.056	0.067	0.062
0.11	0.120	0.118	0.106	0.110	0.107	0.097	0.114	0.095
	0.115	0.124	0.117	0.120	0.092	0.088	0.115	0.100
0.14	0.143	0.124	0.143	0.133	0.129	0.119	0.157	0.114
	0.124	0.158	0.137	0.145	0.115	0.099	0.158	0.127
0.18	0.170	0.164	0.173	0.175	0.165	0.134	0.182	0.157
	0.175	0.148	0.165	0.198	0.144	0.148	0.187	0.160
Incurred				Liver				
1	0.098	0.088	0.110	0.109	0.101	0.130	0.100	0.113
	0.096	0.093	0.113	0.103	0.100	0.117	0.118	0.091
2	0.167	0.154	0.169	0.201	0.174	0.199	0.169	0.170
	0.178	0.153	0.181	0.204	0.162	0.210	0.189	0.180
3	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND = not detected.

Table 3. Collaborative results for sulfadimethoxine in turkey (ppm)

	Laboratory							
Level	1	2	3	4	5	6	7	8
Fortified				Muscle				
0.050	0.052	0.050	0.058	0.060	0.059	0.049	0.040	0.046
	0.055	0.057	0.064	0.055	0.055	0.048	0.051	0.050
0.12	0.135	0.117	0.168	0.159	0.130	0.118	0.116	0.125
	0.124	0.116	0.116	0.156	0.132	0.113	0.128	0.135
0.16	0.155	0.157	0.160	0.192	0.180	0.141	0.158	0.147
	0.165	0.159	0.161	0.194	0.185	0.153	0.176	0.161
0.18	0.195	0.175	0.228	0.232	0.203	0.158	0.177	0.198
	0.176	0.184	0.173	0.220	0.189	0.178	0.206	0.204
Incurred				Muscle				
1	0.055	0.073	0.073	0.026	0.067	0.108	0.069	0.076
	0.053	0.074	0.086	0.029	0.102	0.099	0.063	0.061
2	0.082	0.117	0.104	0.068	0.077	0.114	0.091	0.092
	0.103	0.092	0.088	0.061	0.152	0.126	0.093	0.110
3	ND[a]	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND
Fortified				Liver				
0.06	0.070	0.064	0.063	0.079	0.066	0.063	0.056	0.072
	0.064	0.067	0.065	0.065	0.066	0.071	0.063	0.067
0.10	0.107	0.107	0.105	0.120	0.105	0.112	0.124	0.100
	0.112	0.106	0.095	0.114	0.107	0.102	0.134	0.110
0.15	0.165	0.154	0.171	0.184	0.154	0.152	0.141	0.153
	0.166	0.158	0.157	0.169	0.160	0.156	0.161	0.140
0.19	0.183	0.193	0.228	0.183	0.184	0.187	0.186	0.194
	0.197	0.195	0.190	0.197	0.195	0.193	0.219	0.178
Incurred				Liver				
1	0.062	0.069	0.071	0.085	0.078	0.100	0.094	0.119
	0.070	0.069	0.065	0.087	0.077	0.089	0.100	0.103
2	0.138	0.128	0.143	0.208	0.160	0.227	0.158	0.266
	0.131	0.122	0.127	0.222	0.146	0.183	0.191	0.219
3	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND = not detected.

Results and Discussion

A compilation of the collaborative results is presented in Tables 2–7. Although turkeys were fed medicated diets containing either sulfadimethoxine or sulfaquinoxaline, it was discovered after dosing had begun that the basal diet was contaminated with 3.0 ppm sulfamethazine. Analysts were instructed not to quantitate sulfamethazine in incurred turkey samples but some did nevertheless. Hence, Table 4 contains both qualitative and quantitative responses to sulfamethazine levels in incurred turkey samples. However, Laboratory 7 reported mean values of 0.080 and 0.138 for incurred Samples 1 and 2. These values are higher than would be expected from low level feed contamination. Laboratory 7 reported similar values on both pairs of blind replicates, so the most probable explanation is incorrect labeling of these samples by the originating laboratory. Only one other false positive determination was observed, incurred Sample 1, Laboratory 1.

Outliers (Table 8) were searched for simultaneously, using Dixon's test on the average of the duplicate values for each sample, and a maximum chi-square test on the square differences. Rankings for each sample were determined for these 2 quantities, and a multiple comparison ranking test on the sum of the ranks were together applied to determine outlier laboratories (Table 9). Finally, for each tissue matrix, Dixon's test was applied to the mean of the standardized values for the laboratories. A significance level

Table 4. Collaborative results for sulfamethazine in turkey (ppm)

	Laboratory							
Level	1	2	3	4	5	6	7	8
Fortified				Muscle				
0.07	0.081	0.068	0.082	0.084	0.074	0.065	0.067	0.073
	0.071	0.070	0.068	0.087	0.071	0.071	0.068	0.074
0.09	0.101	0.093	0.110	0.108	0.088	0.089	0.093	0.099
	0.097	0.088	0.099	0.108	0.088	0.088	0.086	0.100
0.15	0.150	0.143	0.159	0.171	0.148	0.145	0.135	0.158
	0.159	0.154	0.163	0.174	0.145	0.142	0.145	0.156
0.20	0.194	0.189	0.192	0.244	0.202	0.174	0.198	0.196
	0.207	0.184	0.198	0.220	0.221	0.187	0.203	0.220
Incurred				Muscle				
1	0.035	ND[a]	0.057	0.057	Tr[b]	Tr	Tr	0.10
	0.108	ND	0.030	Tr	Tr	Tr	Tr	0.10
2	0.01	ND	0.020	ND	Tr	Tr	Tr	ND
	0.01	ND	0.030	Tr	Tr	Tr	Tr	ND
3	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND
Fortified				Liver				
0.07	0.075	0.071	0.078	0.078	0.071	0.083	0.067	0.083
	0.074	0.072	0.082	0.059	0.072	0.065	0.074	0.079
0.11	0.119	0.110	0.116	0.123	0.109	0.117	0.102	0.118
	0.117	0.112	0.123	0.135	0.109	0.110	0.117	0.118
0.16	0.158	0.162	0.168	0.141	0.148	0.168	0.156	0.173
	0.161	0.162	0.175	0.153	0.159	0.167	0.183	0.163
0.20	0.193	0.192	0.197	0.215	0.202	0.219	0.245	0.203
	0.205	0.202	0.186	0.203	0.201	0.209	0.244	0.215
Incurred				Liver				
1	Tr	ND	Tr	Tr	Tr	ND	0.077	Tr
	Tr	ND	Tr	Tr	Tr	ND	0.083	Tr
2	Tr	ND	Tr	Tr	Tr	ND	0.126	Tr
	Tr	ND	Tr	Tr	Tr	ND	0.150	Tr
3	ND	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND = not detected.
[b] Tr = trace amount, not quantitated.

of 0.01 was used to determine outliers for all tests. The ranking test, while yielding no statistically significant differences, did reveal one laboratory usually high, and one laboratory usually low. It should be noted that Laboratory 5 only collaborated on the turkey-related sets.

The complete statistical analysis was repeated deleting the above mentioned outlier values (Table 8). The results changed very little. In a sense, the number of outliers is a measure of the robustness of the procedure. In this case, depending on the statistical test used, only 3 of 1260 individual determinations thus qualified as outliers.

Estimates of the expected coefficient of variation of reproducibility (CV_t) and repeatability (CV_w) at 0.10 ppm for each of the 12 matrices is presented in Table 10. These values were obtained by a regression analysis of the logarithm of variance vs the consensus sample mean. A similar treatment has been previously employed in residue analysis (3). As can be seen from Table 10, the CV_t values are below 15% with the exception of swine liver–sulfadimethoxine. An analysis of variance indicates that of the total variation (reproducibility), on the average, 58 ± 4% is due to within-laboratory variability (repeatability).

The average values for CV_t and CV_w over all sets are 10.5% and 7.7%, respectively.

Although the ideal simultaneous and direct comparison of the TLC, GC/MS, GC, and Tishler

Table 5. Collaborative results for sulfamethazine in swine (ppm)

Level	Laboratory						
	1	2	3	4	6	7	8
Fortified				Muscle			
0.07	0.069	0.071	0.072	0.081	0.062	0.074	0.072
	0.073	0.071	0.084	0.079	0.076	0.074	0.063
0.11	0.113	0.118	0.118	0.107	0.106	0.115	0.107
	0.117	0.117	0.119	0.112	0.114	0.114	0.121
0.16	0.161	0.161	0.169	0.160	0.156	0.162	0.178
	0.167	0.168	0.150	0.153	0.160	0.153	0.167
0.20	0.193	0.191	0.182	0.195	0.197	0.199	0.209
	0.197	0.197	0.241	0.189	0.196	0.203	0.209
Incurred				Muscle			
1	0.135	0.134	0.134	0.114	0.177	0.139	0.150
	0.135	0.139	0.129	0.112	0.162	0.142	0.150
2	0.199	0.195	0.195	0.163	0.231	0.200	0.205
	0.185	0.185	0.183	0.159	0.225	0.204	0.209
3	ND[a]	ND	ND	0.005	ND	ND	ND
	ND	ND	ND	0.007	ND	ND	ND
Fortified				Liver			
0.07	0.076	0.080	0.070	0.098	0.072	0.078	0.059
	0.083	0.072	0.080	0.099	0.081	0.066	0.084
0.09	0.101	0.096	0.086	0.118	0.099	0.103	0.128
	0.098	0.091	0.107	0.104	0.117	0.073	0.091
0.15	0.165	0.144	0.135	0.165	0.132	0.114	0.129
	0.174	0.140	0.143	0.213	0.105	0.132	0.144
0.20	0.190	0.173	0.162	0.207	0.173	0.176	0.184
	0.191	0.187	0.201	0.249	0.189	0.193	0.234
Incurred				Liver			
1	0.090	0.081	0.085	0.113	0.103	0.065	0.059
	0.085	0.083	0.080	0.106	0.117	0.074	0.085
2	0.135	0.153	0.116	0.157	0.203	0.142	0.165
	0.147	0.166	0.117	0.171	0.131	0.130	0.189
3	ND	ND	0.008	ND	ND	0.008	ND
	ND	ND	0.010	ND	ND	0.010	ND

[a] ND = not detected.

procedures was not made because of logistical constraints, some estimates of performance in interlaboratory study can be made. Table 11 provides a comparison of the precision of these methods. The CV_t and CV_w for the GC/MS, GC, and Tishler procedures were calculated by pooling the 4 statistical evaluations in both liver and muscle presented in that study.

The comparison clearly indicates that the TLC and GC/MS procedures have comparable reproducibility and repeatability (about 10–11% and 7.5–8.5%, respectively). Likewise, the GC and Tishler procedures have comparable performance of these 2 parameters (20.5–21.5%, 18.0–18.5%). A review of Tables 1–6 in ref. 1 demonstrates extremely good agreement among the GC, GC/MS, and TLC values. Thus, the differences are present in variability. The GC and Tishler procedures only marginally meet minimum reproducibility criteria established for regulatory residue methods (3). Recovery data for these methods was not reported.

Table 10 also includes a tabulation of the recovery for each data set. Recovery was calculated by linear regression analysis of the consensus mean of the pooled data (across laboratories) at each fortification level vs the known fortification level. The mean recovery across all 12 sets is 95.3%.

Conclusion and Recommendation

Results of this study indicate that the TLC procedure provides data comparable to the GC/MS procedure and is clearly superior to the

Table 6. Collaborative results for sulfadimethoxine in swine (ppm)

	Laboratory						
Level	1	2	3	4	6	7	8
Fortified				Muscle			
0.06	0.057	0.060	0.063	0.074	0.059	0.067	0.059
	0.059	0.063	0.074	0.075	0.065	0.063	0.050
0.10	0.098	0.103	0.096	0.104	0.107	0.098	0.104
	0.099	0.108	0.120	0.097	0.104	0.107	0.113
0.15	0.165	0.157	0.157	0.149	0.150	0.158	0.147
	0.168	0.166	0.161	0.154	0.150	0.162	0.166
0.19	0.200	0.195	0.192	0.189	0.214	0.189	0.204
	0.206	0.202	0.167	0.177	0.199	0.201	0.209
Incurred				Muscle			
1	ND[a]	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
Fortified				Liver			
0.05	0.064	0.054	0.052	0.064	0.057	0.041	0.048
	0.075	0.054	0.052	0.087	0.045	0.052	0.054
0.12	0.140	0.129	0.096	0.162	0.123	0.129	0.164
	0.134	0.122	0.128	0.138	0.150	0.103	0.123
0.16	0.172	0.143	0.124	0.177	0.130	0.139	0.170
	0.162	0.157	0.157	0.226	0.126	0.153	0.225
0.18	0.193	0.180	0.151	0.207	0.138	0.160	0.159
	0.216	0.170	0.181	0.230	0.141	0.139	0.190
Incurred				Liver			
1	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND

[a] ND = not detected.

Table 7. Collaborative results for sulfadimethoxine in duck (ppm)

	Laboratory						
Level	1	2	3	4	6	7	8
Fortified				Muscle			
0.06	0.078	0.072	0.064	0.067	0.076	0.063	0.062
	0.062	0.073	0.064	0.060	0.082	0.063	0.060
0.09	0.094	0.111	0.108	0.134	0.092	0.085	0.089
	0.107	0.116	0.103	0.098	0.121	0.092	0.093
0.16	0.153	0.171	0.163	0.163	0.151	0.157	0.166
	0.151	0.184	0.157	0.153	0.151	0.163	0.154
0.18	0.147	0.203	0.164	0.180	0.201	0.199	0.162
	0.166	0.211	0.148	0.176	0.233	0.246	0.165

continued

Table 7. ***Continued***

Level	Laboratory 1	2	3	4	6	7	8
Incurred				Muscle			
1	0.134	0.152	0.137	0.156	0.180	0.149	0.121
	0.145	0.167	0.145	0.141	0.194	0.165	0.119
2	0.195	0.214	0.196	0.232	0.307	0.252	0.187
	0.204	0.232	0.195	0.235	0.285	0.223	0.211
3	ND[a]	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND
Fortified				Liver			
0.05	0.042	0.062	0.067	0.055	0.061	0.076	0.067
	0.048	0.058	0.056	0.051	0.057	0.054	0.061
0.12	0.120	0.131	0.111	0.144	0.129	0.132	0.137
	0.133	0.138	0.110	0.103	0.134	0.154	0.129
0.15	0.173	0.158	0.144	0.146	0.150	0.163	0.153
	0.177	0.179	0.137	0.128	0.167	0.195	0.162
0.18	0.207	0.201	0.215	0.168	0.191	0.200	0.186
	0.189	0.211	0.198	0.198	0.207	0.240	0.170
Incurred				Liver			
1	0.104	0.095	0.116	0.082	0.115	0.105	0.122
	0.101	0.110	0.110	0.083	0.150	0.112	0.137
2	0.170	0.180	0.146	0.119	0.180	0.157	0.192
	0.165	0.174	0.161	0.109	0.180	0.164	0.191
3	ND	ND	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND	ND	ND

[a] ND = not detected.

Table 8. Outlier identification

Dixon's Test (on the Average of Duplicates)			Chi-Square Test (on the Difference Between Duplicates)		
Matrix–type	Level	Laboratory	Matrix–type	Level	Laboratory
Turkey liver–SMZ	0.20 fortified	7	Turkey muscle–SDM	0.12 fortified	3
Swine liver–SMZ	0.07 fortified	4	Swine muscle–SMZ	0.20 fortified	3
Duck muscle–SDM	0.16 fortified	2	Swine liver–SMZ	incurred No. 2	6

Table 9. Laboratory ranking

Statistic	Laboratory 1	2	3	4	5	6	7	8
Mean	4.30	4.02	4.27	5.01	3.48	4.20	4.26	4.20
Diff. Sq.	3.52	3.45	4.56	4.66	4.38	4.40	4.73	4.49

Tishler colorimetric and Manuel-Steller GC methods. If economic considerations are taken into account, the TLC method is the preferred approach, although the GC/MS method in addition to quantitation provides confirmation as well. Until collaborative data on a revised GC procedure are presented incorporating additional controls on variability (e.g., an internal standard), it is not as reliable as the TLC method. In addition, the TLC method is applicable to 3 drugs in 3 species, whereas the Manuel-Steller GC method is applicable only to sulfamethazine

Table 10. Statistical evaluation of collaborative results

Set	CV_t[a]	CV_w[b]	Rec.[c]
1. Turkey muscle—SQX	14.2	8.2	78.7
2. Turkey liver–SQX	12.5	7.4	80.1
3. Turkey muscle–SDM	10.1	6.8	106
4. Turkey muscle–SMZ	8.2	4.4	98.0
5. Turkey liver–SDM	7.4	5.7	98.3
6. Turkey liver–SMZ	7.5	6.6	102
7. Swine muscle–SMZ	5.8	5.4	97.4
8. Swine muscle–SDM	7.3	5.8	103
9. Swine liver–SMZ	14.4	12.1	86.3
10. Swine liver–SDM	17.0	12.2	92.5
11. Duck muscle–SDM	10.4	7.6	94.7
12. Duck liver–SDM	11.5	9.6	107

[a] Total coefficient of variation (reproducibility) at 0.10 ppm.

[b] Within-laboratory coefficient of variation (repeatability) at 0.10 ppm.

[c] Slope × 100 of linear regression of consensus mean of pooled data at each level vs independently measured value for the fortification level.

Table 11. Reproducibility and repeatability of 4 methods for sulfonamide analysis

Statistic	TLC	GC/MS[a]	GC[a]	Tishler[a]
CV_t	10.5	10.9	21.6	20.5
CV_w	7.7	8.5	18.5	18.0

[a] Average of all statistical evaluations of CV_t, CV_w presented in ref. 1, Table 7.

in swine. For these reasons it is recommended that the thin layer chromatographic procedure be adopted official first action.

Acknowledgments

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Collaborators: John Patterson, Agriculture Canada, Guelph, Ontario; Tracy Hunter, Consolidated Laboratories of Virginia, Richmond, VA; Mary Leadbetter, FDA, Beltsville, MD; Blaise D. Willis, FSIS, Athens, GA; Colen Kao, FSIS, San Francisco, CA; Fred Mitchell, FSIS, St. Louis, MO; Karen E. Soroka and Sharon H. Thomas, FSIS, Beltsville, MD; Leonard Gonzales, Hoffmann-La Roche, Inc., Nutley, NJ.

For protocol design and statistical evaluation: Anthony J. Malanoski, Jon McNeal, Richard Ellis, FSIS, Washington, DC.

For sample collection and preparation: Kevin Curry, Paul Corrao, Jean Jackson, Warren Lishear, FSIS, Beltsville, MD.

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PRESERVATIVES

Gas-Liquid Chromatographic Determination of Dehydroacetic Acid in Squash and Wine

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A gas-liquid chromatographic procedure has been developed for the determination of the preservative dehydroacetic acid (DHA) in frozen cut squash and white wine. The cleanup procedure uses the acidic character of DHA to enhance separation from interfering substances. Recoveries were 93-104% from squash fortified at 30, 65, and 130 ppm and 96-99% from wine fortified at 50, 100, and 200 ppm. The method can be used to establish that the amount of DHA used in squash does not exceed the permissible level of 65 ppm.

The interest in food applications of dehydroacetic acid (DHA) (3-acetyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione) is demonstrated by the number of publications that describe methods for its determination in wine (1-3), beer (4), cheese (1, 5), and squash (6, 7). In the United States, authorized use of DHA is limited to frozen cut squash at 65 ppm (8).

Chromatographic and spectrophotometric techniques have been used to determine DHA in food. A variety of food preservatives, including DHA, were subjected to gas-liquid chromatography (GLC) (9) and high pressure liquid chromatography (HPLC) (10). The GLC quantitation was preceded by a liquid/liquid cleanup procedure, whereas steam distillation was used to prepare the sample for HPLC. Ultraviolet (UV) spectroscopy was used by Ramsey (5) to quantitate DHA in cheese. The procedure in the *Food Additives Analytical Manual* (7) uses acid-base liquid/liquid partitioning to prepare the food extracts for UV quantitation at 307 nm. Since no chromatographic separation precedes the spectrophotometric quantitation, interference by other UV-absorbing compounds is likely, and results would need to be interpreted with caution. Piwowar (6) overcame this deficiency to some extent by interposing a thin layer chromatographic (TLC) cleanup step between the liquid/liquid partitioning and the UV quantitation. Although the TLC step increased the reliability of the results, the procedure required a greater number of manipulative steps.

GLC techniques (1, 9) have been successfully used with food matrices other than squash; therefore, this basic approach was selected for this work. The combination of acid-base liquid/liquid extraction and GLC provides the specificity required to determine DHA in food samples. Squash was selected because the level of use is limited by regulation. The work was extended to include wine because there is concern that DHA might be misused as a preservative in this product as indicated by Haller and Junge (2).

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METHOD

Reagents

(a) *Methylene chloride.*—Distilled-in-glass (Burdick and Jackson, Muskegon, MI 49441).

(b) *2,4-Diisopropylphenol (DPP) standard solutions.—Stock solution.*—About 2 mg/mL. Transfer 200 mg DPP (Eastman Organic Chemicals, Rochester, NY 14692) to 100 mL volumetric flask. Dilute to volume with methylene chloride. *Working standards.*—800, 400, 200, and 100 μg/mL. Dilute appropriate aliquots of stock solution with methylene chloride.

(c) *DHA standard solutions.—Stock solution.*—About 2 mg/mL. Quantitatively transfer known quantity of DHA (Aldrich Chemical Co., Inc., Milwaukee, WI 53233) (ca 200 mg) to 100 mL volumetric flask. Add 50 mL methylene chloride; dissolve in and dilute to volume with methylene chloride. *Working standards.*—1.6 mg/mL, 1.0 mg/mL, 500 μg/mL, and 250 μg/mL. Serially dilute aliquots of stock solution with methylene chloride.

Apparatus

(a) *Gas chromatograph.*—Tracor Model 560 (Tracor Instruments, Austin, TX 78704) equipped with flame ionization detector. Operating conditions: carrier gas ultra high purity helium at 50-60 mL/min or nitrogen at 30 mL/min

passed through oxygen trap; hydrogen 30 mL/min; air 300 mL/min (all gases passed through activated 13X molecular sieve); temperature program: 110–150°C at 5°/min followed by 15 min hold; inlet temperature 220°C; detector temperature 280°C; electrometer 4×10^{-12} to 6.4×10^{-10} amp full scale; 1 mV recorder, 3.2 mm/min chart speed.

(b) *Chromatographic column.*—91.4 cm × 2 mm id glass, packed with 5% Carbowax 20M–terephthalic acid on Chromosorb W-HP.

(c) *Mixer.*—Sorvall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, CT 06850) equipped with 500 mL cups.

(d) *Centrifuge.*—Sorvall Model RC-3 (Ivan Sorvall) equipped to hold 250 mL polyethylene centrifuge bottles with screw caps.

Sample Preparation and Cleanup

White wine.—Place 100–300 mL white wine in 1 L side-arm vacuum flask. Add 51 mm magnetic stirring bar. Stopper, begin stirrer, and cautiously apply vacuum until sample is degassed. To degas, apply vacuum until no further bubbles are observed. Weigh 50 g degassed white wine into 125 mL separatory funnel. Add 10 mL 3N NaOH and 10 mL methylene chloride and shake 1 min. (In all subsequent extraction steps, shake both wine and squash 1 min.) Centrifuge if necessary to separate layers. Transfer methylene chloride layer to stoppered 60 mL separatory funnel. (Emulsion should be left with aqueous layer.) Extract methylene chloride with two 10 mL portions of 1.2N NaOH. Combine aqueous layers in 125 mL separatory funnel. Discard methylene chloride layer. Add 15 mL 5N HCl to aqueous extract. Add 10 mL methylene chloride, shake, and centrifuge if necessary to separate layers. Transfer methylene chloride layer to stoppered 25 mL graduated cylinder. As before, if slight emulsion persists, leave emulsion with aqueous layer. Re-extract aqueous layer with two 5 mL portions of methylene chloride. Centrifuge if necessary after each extraction to separate layers. Combine methylene chloride extracts in 25 mL graduated cylinder. After last extraction, combine emulsion with methylene chloride in 25 mL graduated cylinder; stopper and shake cylinder. Using pipet, carefully remove aqueous phase. Add known volume of internal standard solution equivalent to quantity of DPP which is ca one-fourth of weight of DHA anticipated in sample. Dilute to 25 mL with methylene chloride and shake.

Squash.—Accurately weigh 40 g finely chopped squash to nearest 0.1 g in 500 mL Omni-Mixer cup. Add ca 80 mL methylene chloride and 5 mL 2N HCl. Blend sample 5–7 min at low speed. Using powder funnel, pour sample into 250 mL plastic centrifuge bottle. Centrifuge 10 min at ca 1500 rpm. Remove lower methylene chloride layer with 50 or 100 mL pipet and transfer to 250 mL separatory funnel labeled No. 1. Wash mixer cup with 40–50 mL methylene chloride. Transfer solution to centrifuge bottle, using powder funnel as before. Add 5 mL 0.5N HCl, shake, and centrifuge 10 min at ca 1500 rpm. Add methylene chloride layer to separatory funnel No. 1, using 50 or 100 mL pipet. Repeat extraction with another 40–50 mL methylene chloride.

Extract methylene chloride solution in separatory funnel No. 1 with 25 mL 2N NaOH. Transfer methylene chloride to another 250 mL separatory funnel labeled No. 2 and transfer water layer to 125 mL separatory funnel labeled No. 3 containing 30 mL 5N HCl. Extract methylene chloride layer in separatory funnel No. 2 with 25 mL 1N NaOH. Transfer methylene chloride to another 250 mL separatory funnel labeled No. 4 and transfer water layer to separatory funnel No. 3. Again extract methylene chloride solution in separatory funnel No. 4 with 25 mL 1N NaOH. Discard methylene chloride layer and transfer water layer to separatory funnel No. 3. Discard extracts contained in 250 mL funnels. Add 10 mL methylene chloride to aqueous solution in separatory funnel No. 3. Shake and transfer methylene chloride layer to stoppered 25 mL graduated cylinder, leaving behind any emulsion. Re-extract aqueous layer with two 5 mL portions of methylene chloride. After last extraction, let solution in separatory funnel separate for 2 min; transfer methylene chloride layer and any emulsion to same 25 mL cylinder. Stopper and mix. Using pipet, carefully remove aqueous phase. Add known volume of internal standard solution which will deliver quantity of DPP that is ca one-fourth of weight of DHA anticipated in sample. Dilute to 25 mL with methylene chloride and shake.

Recovery Studies

White wine.—Weigh 50 g degassed white wine that does not contain DHA into 125 mL separatory funnel. Add enough DHA so that concentration of DHA in sample is between 50 and 200 ppm. Proceed as directed under *Sample Preparation and Cleanup—White wine.*

Squash.—Weigh 40 g finely chopped squash into 500 mL Omni-Mixer cup. Add enough

DHA so that concentration of DHA in sample is between 30 and 130 ppm. Proceed as directed under *Sample Preparation and Cleanup—Squash.*

Standard Solutions

Transfer known volumes of DHA stock standard solution to 3 25 mL graduated cylinders equipped with ground-glass stoppers. Range of quantities of DHA in graduated cylinders should bracket anticipated quantity in sample. Select amounts of stock solution which will give required quantities in volumes of ≤15 mL. To each, add same volume of DPP stock standard solution that was added to sample extract. Dilute each to ca 25 mL with methylene chloride, stopper, and mix.

Gas Chromatography

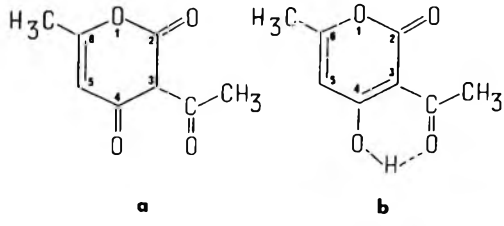
Inject ca 3 μL aliquots of each standard repeatedly until relative peak heights differ by no more than ca 2%. Two or 3 injections are usually required. To prepare calibration curve, plot quotient of DHA peak height divided by DPP peak height as ordinate vs. quantity of DHA (μg) in standard as abscissa. Use least squares treatment to determine best straight line through points.

Inject ca 3 μL aliquots of sample solution repeatedly until relative peak heights differ by no more than ca 2%. Determine DHA/DPP peak height ratio and enter value on standard curve to determine total weight of DHA (μg) in sample. Calculate DHA by following formula:

$$\text{DHA in sample, ppm} = \text{DHA in extract, } \mu g / \text{sample weight, g}$$

Results and Discussion

The complete method incorporates an acid-base liquid/liquid partitioning step followed by GLC with quantitation by the flame ionization detector. The first step is based upon the acidity of the carbon-hydrogen bond at C-3 of DHA (a), which is activated by 3 adjacent carbonyls. DHA can also exist in different enolic forms, one of which is depicted by (b).



a b

Whether the conjugate base of DHA occurs by ionization of the carbon-hydrogen bond in form (a) or by ionization of the oxygen-hydrogen bond in one of the enolic forms, the stability of the resulting anion is enhanced because the negative charge is distributed over 3 oxygen atoms and 1 carbon atom. The partitioning behavior of DHA between water and methylene chloride can be controlled because the degree of ionization of DHA can be varied from 0 to 100% within the accessible pH range of aqueous solutions.

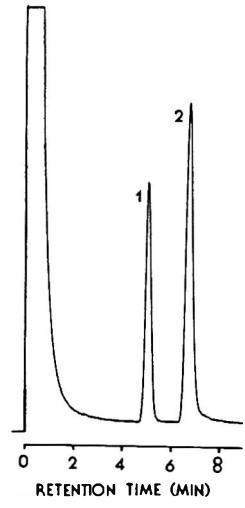


Figure 1. Gas chromatogram of internal standard DPP (peak 1, 0.075 μg on-column) and DHA (peak 2, 0.3 μg on-column).

As shown in Figure 1, DHA chromatographs on a Carbowax 20M-terephthalic acid column as a single, symmetrical peak. The internal standard is added to compensate for variations in extract and injection volumes. A linear relationship exists between the normalized peak height and the concentration of DHA over the range described in the analytical method.

Table 1 shows that good recoveries were obtained with squash and wine. Squash was spiked at levels of 30, 65, and 130 ppm and wine at levels of 50, 100, and 200 ppm. The cleanup procedure, which takes advantage of the acidic character of DHA, provided sample extracts that were free of interferences. The average recovery of added DHA ranged from 93 to 104%. Extraction of red wine was difficult because of the formation of stable emulsions; therefore, this food matrix was not studied further. No survey of squash and wine was undertaken because no DHA was indicated on the labels of a large number of different brands of these products.

Table 1. Recovery of DHA from fortified squash and white wine

No. detns	Added, ppm	Mean rec., %	Std dev.
	Squash		
7	30	103	2.7
8	65	104	2.4
8	130	93	3.5
	White Wine		
5	50	97	1.5
5	100	99	1.0
5	200	96	2.3

The proposed method can be used to determine that DHA is not used in quantities exceeding the permissible level of 65 ppm in squash (8) and possible misuse in white wine. The method is simple and rapid because it uses a straightforward liquid/liquid partitioning cleanup followed by GLC quantitation which does not require derivatization. Although the retention times for DPP and DHA vary with different column packing preparations, the order of elution remains unchanged. With the calibration procedure described here, the method has consistently given reliable results in the 3 years it has been used. With slight modification, the procedure could be used to detect unauthorized use of DHA in a variety of other foods.

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MICROBIOLOGICAL METHODS

Enumeration of Coliforms in Nonfat Dry Milk and Canned Custard by Hydrophobic Grid Membrane Filter Method: Collaborative Study

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A collaborative study was conducted in 14 laboratories to evaluate the performance of the hydrophobic grid membrane filter method against the official final action method, 46.016, for enumeration of coliforms in foods. Twelve artificially contaminated samples of nonfat dry milk and canned custard were analyzed by each laboratory. The random errors (S_r^2) associated with the hydrophobic grid membrane filter method were significantly lower than those of the official method, and the counts obtained by the new method fell within the 95% confidence interval of the reference method. The coliform counts obtained by the hydrophobic grid membrane filter method did not differ significantly from those obtained by the reference method as measured by a 3-way analysis of variance. The new method has been adopted official first action.

The hydrophobic grid membrane filter (HGMF) was first described in 1974 by Sharpe and Michaud (1). It consisted of a membrane filter on which had been imprinted a grid pattern formed from a nontoxic hydrophobic material. The hydrophobic lines acted as barriers to the lateral spread of colonies, thus subdividing the surface of the membrane into a large number of separate growth compartments of known and equal size. In 1975, Sharpe and Michaud presented arguments and data to support the treatment of the HGMF as a most probable number counting device (2). This feature extended the counting range of the hydrophobic grid membrane filter well beyond the actual number of growth compartments on the filter surface.

These early studies were carried out on pure culture suspensions and on water samples. In 1979, Sharpe et al. (3) reported that a wide variety of food products could be made to filter through a 0.45 μm membrane filter in quantities of 0.1 g per filter or more. Further studies on the membrane filtration of foods, published in 1980 (4) and 1982 (5), showed that large enough quantities of almost any food could be filtered through a 0.45 μm hydrophobic grid membrane filter to produce a lower limit of detection equivalent to conventional enumeration methods.

In 1982, Brodsky et al. (6) described an overnight, single-step enumeration procedure for total coliforms that made use of the hydrophobic grid membrane filter. The method they developed had several advantages over conventional coliform methods, including the following: a final result was available in 24 h; soluble inhibitors and interfering substances present in the sample could be rinsed through the filter; the need to make several serial 10-fold dilutions for most samples was eliminated, because the counting range of the HGMFs used in the study was approximately 4800; and the use of a resuscitation procedure for the enumeration of potentially injured coliforms was facilitated.

The purpose of this collaborative study was to compare the performance of the hydrophobic grid membrane filter method against the official final action method, **46.016**, for the enumeration of coliforms.

Collaborative Study

Microbiologists from 14 different laboratories, served as collaborators. Each collaborator received a complete set of instructions, a data sheet, a set of inoculum tubes numbered 1–12, 100 g nonfat dry milk (NFDM), and 140 g canned vanilla custard. Special materials and media required for the hydrophobic grid membrane filter method were provided by the Associate Referee;

This report of the Associate Referee was presented at the 96th Annual International Meeting of the AOAC, Oct. 25–28, 1982, at Washington, DC.
Received July 14, 1982.
The recommendation of the Associate Referee was approved by the General Referee and Committee F and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1982) 66, March issue.

Table 1. Description of inoculum mixtures

Mixture	Inoculum	No. of strains
1	*Escherichia coli*	5
2	*Enterobacter aerogenes*	1
	Enterobacter agglomerans	1
3	*E. coli*	5
	E. aerogenes	1
	E. agglomerans	1
	Citrobacter freundii	2
4	*Serratia marcescens*[a]	1

[a] Non-lactose fermenter.

all other materials were furnished by the collaborating laboratories.

Four inoculum mixtures were prepared in NSB preservation medium (7) from overnight tryptic soy agar slant cultures. The contents of each mixture are described in Table 1. The inoculum densities of the mixtures were adjusted to provide a range of coliform levels when a consistent volume of mixture was used to inoculate a 10 g sample portion. Each mixture was dispensed aseptically into screw-cap tubes; the tubes were numbered according to the scheme outlined in Table 2, stored at room temperature, and shipped under ambient temperature conditions. Several tubes from each mixture were retained by the coordinating laboratory.

All collaborators were instructed to begin their analyses on the same date by weighing 6 samples of 10 g from the NFDM (numbered 1–6) and from the custard (numbered 7–12). Each 10 g sample was inoculated with 1.0 mL from the inoculum tube of the corresponding number and analyzed by both methods immediately following inoculation. On the designated set-up date, the coordinating laboratory performed 9 replicate counts from each inoculum mixture (3 replicates from each of 3 tubes), using the HGMF method, to determine the approximate coliform density in each mixture. The results of these control counts, expressed as coliforms per gram of sample, are shown in Table 2.

Table 2. Description of collaborative samples

Sample	Food	Mixture	Coliforms/g[a]
1	NFDM[b]	2	8.5×10^3
2	NFDM	1	9.6×10^4
3	NFDM	3	2.3×10^3
4	NFDM	3	2.3×10^3
5	NFDM	1	9.6×10^4
6	NFDM	2	8.5×10^3
7	custard	4	$<10^2$[c]
8	custard	2	8.5×10^3
9	custard	4	$<10^2$[c]
10	custard	1	9.6×10^4
11	custard	2	8.5×10^3
12	custard	1	9.6×10^4

[a] Based on 9 replicate filtrations of each mixture after 7 days storage at 22–25°C.
[b] Nonfat dry milk.
[c] Inoculated with non-lactose fermenting bacteria.

Each collaborator analyzed all 12 samples by both the HGMF method described below and the official method, **46.016,** using the dilution series specified in the set of instructions. The collaborators calculated the most probable number (MPN) of coliforms per gram for both methods and reported both the unconverted count (or number of gas-positive tubes) and the MPN/g obtained by both methods for each sample.

The procedures outlined in the proposed method and in the official method were followed except that 1.0 mL of the 10^{-2} dilution of each sample was filtered, and the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions were used to inoculate the 3-tube MPN series to cover the full range of coliform counts anticipated in the 12 samples. Gas-positive tubes were subcultured to BGLB only, instead of BGLB and EC as specified in method **46.016.** The 3 consecutive dilutions used to calculate the 3-tube MPN index were chosen as described in *Standard Methods for the Examination of Dairy Products,* 14th edition (8).

Enumeration of Coliforms in Selected Foods
Hydrophobic Grid Membrane Filter Method
Official First Action

(Applicable to nonfat dry milk and canned custard)

46.D04 ***Principle***

The hydrophobic grid membrane filter (HGMF) method uses a membrane filter implanted with hydrophobic material in a grid pattern. These hydrophobic lines act as barriers to the spread of colonies, thereby dividing the membrane filter surface into a number of separate compartments of equal and known size. The number of squares occupied by colonies is enumerated and converted to a most probable number value of organisms by using the formula given below.

46.D05 ***Apparatus***

(a) *Hydrophobic grid membrane filter (HGMF).* —Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material

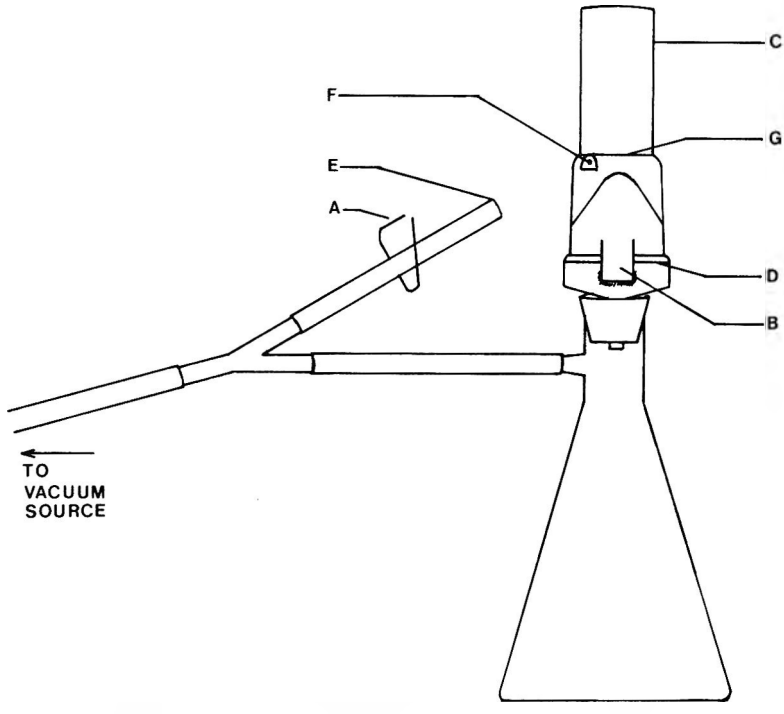

Figure 46:D2. Schematic of hydrophobic grid membrane filtration unit.

in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(b) *Filtration units for HGMF.*—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd) or equiv. meets these specifications.

(c) *Pipets.*—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory.

(d) *Blender.*—Waring Blendor, or equiv., multispeed model, with low-speed operation at 12 000–14 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(e) *Vac. pump.*—Water aspirator vac. source is satisfactory.

(f) *Manifold or vac. flask.*

46.D06 *Culture Media and Reagents*

(a) *Peptone/Tween 80 diluent.*—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L H_2O. Dispense enough vol. into diln bottles to give 90 ± 1 mL after autoclaving 15 min at 121°.

(b) *M-FC agar.*—10.0 g tryptose, 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 5.0 g NaCl, 12.5 g lactose, 1.5 g bile salts No. 3, 0.1 g aniline blue, and 15.0 g agar dild to 1 L with H_2O (M-FC Agar, Difco 0677 is satisfactory). Heat to boiling. Temper to 50–55°. Adjust pH to 7.4 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface dry plated medium before use.

46.D07 *Sample Preparation*

Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (a) and blend 2 min at low speed (12 000–14 000 rpm). Use this 1:10 diln to prep. further serial dilns if required.

46.D08 *Analysis*

(See Fig. **46:D2.**) Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Pull forward on latch B, and rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward and clamp unit shut by pressing with thumb on latch B.

Aseptically add ca 15–20 mL sterile H_2O or diluent to funnel. Pipet 1.0 mL of 1:10 sample

diln into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL H_2O or diluent to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

Open clamp A. Release latch B, and rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried M-FC agar (**b**). Avoid trapping air bubbles between filter and agar. Incubate 24 ± 2 h at 35°.

Count all squares contg one or more blue colonies. Include any shade of blue. Score each square as either occupied or empty. Convert occupied square count to MPN with the formula

$$\text{MPN} = [N \log_e (N/(N - x))]$$

where N = total number of squares and x = number of occupied squares. Multiply by diln factor and report as MPN of total coliform bacteria/g.

Results and Discussion

The data reported by the collaborators for both methods were checked for correctness of the most probable number indices and dilution factor calculations, rounded to 2 significant figures, and converted to $\log_{10}$ values for statistical analysis. The hydrophobic grid membrane filter data are displayed in Table 3 and the 3-tube MPN data are in Table 4.

Three collaborators reported significant method deviations for some or all of the 12 samples. Collaborator 3 stored the inoculum tubes at 4–6°C on receipt, instead of at room temperature as specified. After 2 days, the tubes were placed at room temperature until the scheduled setup date. This resulted in erratic and unpredictable changes in the bacterial population, with total die-off in some tubes and abundant growth in others. As a result, it was necessary to exclude these data from all of the statistical analyses. Collaborator 7 analyzed Samples 1–6 inclusive as instructed and then disinfected the filtration units in 5% chlorine. Due to inadequate rinsing of residual chlorine from the filtration units, no growth was obtained by this collaborator from Samples 7–12 by the HGMF method. Collaborator 12 reported a deviation in filtering technique that produced a very uneven distribution of occupied squares on the grid matrix. Because the most probable number treatment of the occupied square count presupposes a reasonably uniform distribution of the inoculum over the surface of the filter, the data from this collaborator were excluded from statistical analysis.

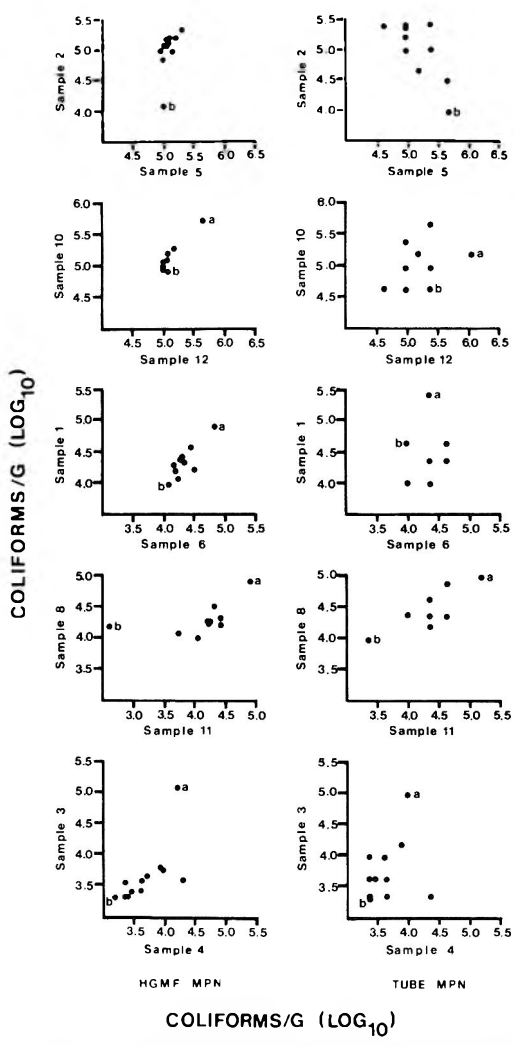

Figure 1. XY control charts for hydrophobic grid membrane filter and 3-tube most probable number methods: a, Collaborator 9; b, Collaborator 13.

Four samples were lost due to breakage (one sample) or leakage (3 samples) of inoculum tubes. In these instances, data from the other member of the sample pair were excluded from the determination of random error and bias. Two collaborators (8 and 10) isolated coliforms in significant numbers by both methods from a negative control sample. Data from these collaborators were excluded from statistical analyses, but were included in a scatter plot comparison of overall coliform recovery by both methods, because the counts obtained from the other samples all fell in the expected ranges.

Table 3. Recovery of coliforms by the hydrophobic grid membrane filter method: collaborative results

	Coliform count/g (log_{10}) for samples:											
Coll	1	2	3	4	5	6	7	8	9	10	11	12
1	4.40	5.18	3.66	3.70	5.20	4.30	<2.00	4.32	M[a]	5.18	4.43	5.08
2	4.56	5.18	3.81	3.93	5.11	4.46	<2.00	4.51	<2.00	5.04	4.32	M
3[b]	3.61	>5.96	4.20	3.40	<2.00	5.00	<2.00	<2.00	<2.00	5.04	>5.96	5.08
4	4.04	5.04	3.41	3.46	5.04	4.23	<2.00	4.08	<2.00	5.34	4.23	M
5	4.32	4.97	3.56	3.36	5.15	4.32	<2.00	4.26	<2.00	4.93	4.26	5.00
6	4.34	5.30	3.58	3.62	5.30	4.30	<2.00	4.26	<2.00	5.26	4.26	5.18
7[c]	4.15	4.83	3.32	3.18	4.99	4.04	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
8	4.20	4.97	3.74	3.97	4.95	4.49	3.26	4.28	<2.00	5.00	4.20	5.00
9[d]	4.88	M	5.08	4.20	5.57	4.82	<2.00	4.88	<2.00	5.72	4.91	5.65
10	4.18	5.08	3.61	4.30	4.99	4.20	<2.00	4.20	4.32	4.94	4.43	5.00
11	4.28	5.15	3.34	3.40	5.04	4.15	<2.00	4.00	<2.00	5.20	4.04	5.08
12[b]	4.08	4.79	3.54	3.41	4.67	4.26	<2.00	4.20	<2.00	4.84	4.18	4.93
13[d]	3.95	4.08	3.34	3.34	5.00	4.08	<2.00	4.18	<2.00	4.89	2.60	5.08
14	4.15	5.11	3.43	3.60	5.08	4.23	<2.00	4.23	<2.00	5.08	4.23	5.00

[a] Missing value; sample lost.
[b] Results were excluded from all statistical analyses. See text.
[c] Results were excluded from all statistical analyses. Results from Samples 2–5 were included in Figure 2. See text.
[d] Results were excluded from all statistical analyses but were included in Figure 2. See text.

Table 4. Recovery of coliforms by the 3-tube most probable number procedure: collaborative results

	Coliform count/g (log_{10}) for samples:											
Coll.	1	2	3	4	5	6	7	8	9	10	11	12
1	4.36	5.38	3.97	3.36	4.97	4.36	<2.48	4.36	M[a]	5.66	4.63	5.38
2	4.36	5.38	3.63	3.63	4.97	4.36	<2.48	4.63	<2.48	5.38	4.36	M
3[b]	3.36	5.66	4.36	4.18	<2.48	5.66	<2.48	<2.48	2.56	>6.04	4.63	>6.04
4	4.36	4.97	3.36	3.63	4.97	4.36	<2.48	4.36	<2.48	5.38	4.36	M
5	4.36	4.63	3.63	3.36	5.18	4.63	<2.48	4.36	<2.48	4.97	4.36	4.97
6	4.63	4.97	3.97	3.36	5.38	4.63	<2.48	4.36	<2.48	4.97	3.97	5.38
7[c]	<2.48	5.32	3.36	3.36	4.97	<2.48	<2.48	<2.48	<2.48	4.63	<2.48	4.36
8	4.36	5.38	4.18	3.88	5.38	4.63	3.36	4.36	<2.48	4.63	4.36	4.97
9[d]	5.38	M	4.97	3.97	5.66	4.36	<2.48	4.97	2.56	5.18	5.18	6.04
10	3.97	5.38	3.36	4.36	4.63	4.36	<2.48	4.36	4.18	4.63	3.97	4.63
11	4.36	4.46	3.63	3.46	5.66	4.36	<2.48	4.18	<2.48	5.38	4.36	4.97
12[b]	<2.48	5.38	3.36	3.88	4.36	2.56	<2.48	<2.48	<2.48	5.18	<2.48	6.04
13[d]	4.63	3.97	3.30	3.36	5.66	3.97	<2.48	3.97	<2.48	4.63	3.36	5.38
14	3.97	5.18	3.97	3.59	4.97	3.97	<2.48	4.88	<2.48	5.18	4.63	5.18

[a,b,c,d] See Table 3.

Table 5. Comparison of random and systematic error associated with the hydrophobic grid membrane filter (HGMF) and 3-tube most probable number (MPN) methods

			Random error (S_r^2)			Systematic error (S_b^2)	
Pair	Sample	DF[a]	HGMF	MPN	F[b]	HGMF	MPN
I	2 & 5	8	0.003	0.083	27.667[c]	0.005	−0.036
II	10 & 12	5	0.001	0.025	25.000[c]	0.005	0.007
III	1 & 6	7	0.003	0.003	1.000	0.007	0.019
IV	8 & 11	7	0.003	0.015	5.000[c]	0.008	0.011
V	3 & 4	8	0.004	0.024	6.000[c]	0.017	−0.004

[a] Degrees of freedom.
[b] $F = [S_r^2 (MPN)]/[S_r^2 (HGMF)]$.
[c] Statistically significant ($\alpha = 0.05$).

The remaining data were checked for the presence of outlying laboratories by the rank sum test described by Youden (9). Collaborators 9 and 13 were outliers for the HGMF data, with 9 producing consistently high counts relative to the other laboratories and 13 being consistently low. These same laboratories were also the highest and lowest ranked respectively for the 3-tube most probable number method, although 13 was not an outlier. The XY control charts for both methods (see Figure 1) confirmed the tendency of both these collaborators to be positioned away from the main cluster of data for most sample pairs. Neither collaborator reported a method deviation, so it is likely that the exceptional results obtained by these laboratories were a consequence of the environmental (especially, temperature) conditions to which the samples may have been exposed in transit. The data from Collaborators 9 and 13 were excluded from the determination of random error and bias, but were included in the comparison of overall coliform recovery by the 2 methods.

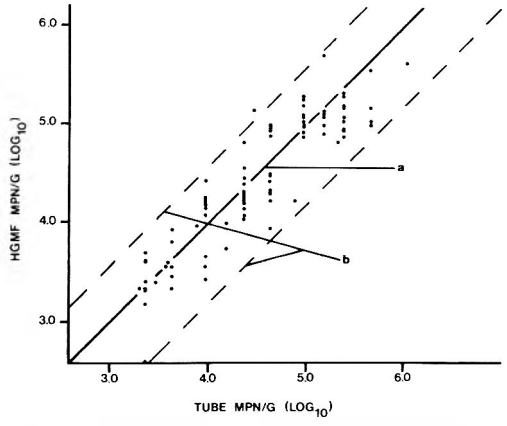

Figure 2. Scatterplot of coliforms/g for hydrophobic grid membrane filter and 3-tube most probable number (MPN) methods: a, line of theoretical equivalence; b, approximate upper and lower 95% confidence interval of 3-tube MPN counts.

The data from each sample pair were evaluated to determine the random error (S_r^2) and bias (S_b^2) for the 2 methods (9). These results are summarized in Table 5. The S_r^2 was consistently lower for the hydrophobic grid membrane filter method than for the reference method, and an F-test was performed to assess the statistical significance of this difference. The random error was determined to be significantly lower for the HGMF method than for the official method in 4 of the 5 sample pairs ($P < 0.05$).

The repeatability and reproducibility standard deviations and coefficients of variation were calculated separately for each method and food and are displayed in Table 6. A 3-factor analysis of variance was carried out separately for each food to evaluate laboratory, sample, and method interactions. No significant interactions were found between methods and laboratories or between methods and samples for either food, nor did the methods differ overall ($P = 0.05$). Highly significant ($P < 0.01$) differences were found between samples, as expected. Also, significant ($P < 0.05$) differences were found between laboratories with the nonfat dry milk and significant ($P < 0.05$) laboratory-sample interaction was found with the custard. These differences were likely due to differences in environmental conditions to which each set of samples was exposed in transit. The quantitative coliform recoveries obtained by the 2 methods were also compared graphically by preparing a scatter plot of the data that had not been exposed to method deviations. As shown in Figure 2, all but one of the counts obtained by the hydrophobic grid membrane filter method fell within the 95% confidence interval of the corresponding 3-tube most probable number result.

Table 6. Precision estimates associated with hydrophobic grid membrane filter (HGMF) and 3-tube most probable number (MPN) methods

	Nonfat dry milk		Custard	
Parameter	HGMF	MPN	HGMF	MPN
Mean	4.3283	4.3498	4.6462	4.7923
σ_o[a]	0.0731	0.2857	0.0690	0.1834
σ_x[b]	0.1425	0.2857	0.1501	0.2173
CV for σ_o[c], %	1.69	6.57	1.48	3.83
CV for σ_x[d], %	3.29	6.57	3.23	4.53

[a] Repeatability standard deviation.
[b] Reproducibility standard deviation.
[c] Repeatability coefficient of variation.
[d] Reproducibility coefficient of variation.

Comments received from collaborators about the hydrophobic grid membrane filter method were generally favorable, except that densely populated filters were tedious to count. Laboratory 9 made use of a modified Quebec-type colony counter developed by A. N. Sharpe (personal communication) that had been provided for another study. They reported that this device facilitated counting. Collaborators 7 and 12 could not recover coliforms successfully from samples spiked with Mixture 2, using the official method, although they did obtain coliform counts with the hydrophobic grid membrane filter method. Collaborator 12 re-analyzed the 12 samples 10 days after the initial set up, using inoculum left over from the first analysis. Coliforms were recovered from all of the appropriate samples by the HGMF method, although significant die-off of the coliform population had occurred. However, the 3-tube MPN procedure was only able to recover coliforms from 3 of these old inocula (Samples 2, 3, and 5). Collaborator 11 reported LST and BGLB counts from samples spiked with Mixture 2 could only be made after 48 h incubation, although these same samples produced lactose-positive colonies on the M-FC agar after 24 h. Mixture 2 consisted of one strain each of *Enterobacter aerogenes* and *E. agglomerans*. We have found these strains to be relatively slow lactose-fermenters. It would appear, from these observations, that the HGMF method is capable of detecting slow lactose fermenters even when these grow poorly in LST.

The quantitative results reported here, together with the supplementary observations mentioned above, show that the hydrophobic grid membrane filter method is as effective as the 3-tube MPN procedure for the enumeration of coliforms in the 2 foods tested, as well as being subject to significantly less random error than the 3-tube MPN method. The original coliform HGMF method described by Brodsky et al. (6) included a 2-h resuscitation step during which the inoculated HGMFs were incubated on a nonselective medium prior to being placed on the M-FC agar. Further work carried out in our laboratory has shown that in the majority of cases a resuscitation step does not change the HGMF coliform count significantly relative to the tube MPN procedure although it does increase the coliform count relative to an unresuscitated HGMF count (10). Preliminary experiments carried out by the coordinating laboratory showed that incorporation of a resuscitation step made no measurable difference to coliform counts obtained from 10-day-old NSB. Therefore, no provision was made in this collaborative study for a resuscitation step.

Recommendation

It is recommended that the proposed hydrophobic grid membrane filter method for enumeration of coliforms in nonfat dry milk and canned custard be adopted official first action.

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MYCOTOXINS

Coupled-Column System for Quantitating Low Levels of Aflatoxins

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The combination of Styragel and Florisil columns allows recovery of aflatoxins at low levels. Two switching valves between the columns reduce the analysis time and exposure to solvents. Florisil is modified with oxalic acid, allowing recovery of aflatoxins at levels below 500 pg. The method is useful for contaminated corn and peanut meal samples.

Methods for quantitating aflatoxins are numerous. However, accepted methods tend to be matrix-dependent, requiring new techniques for each new matrix. As part of a project investigating the air quality at a plant producing refuse-derived fuel (RDF), a method for separating mycotoxins from a complex matrix has been developed (1). These techniques have been incorporated into an analytical method for determining aflatoxins, employing laser-induced fluorescence on thin layer chromatographic (TLC) plates (2).

The chromatographic separations that have been developed include exclusion/partitioning on Styragel and a modified Florisil adsorbent which uses a unique column design. These 2 chromatographic systems have been combined to minimize the time and extent of sample handling involved.

The preparation and use of a Styragel column has been described (1) and an understanding of solvent effects which enhance separation was proposed. Florisil has been suggested for use in the determination of aflatoxins (3, 4). The adsorbent was deactivated with acetic acid and water. However, this modification was designed for relatively high levels (>1 ng) of aflatoxins and we could not recover aflatoxins added to a Florisil column at levels less than 500 pg.

A new Florisil modification using oxalic acid and a unique column design allows recovery at low levels of aflatoxins. The Styragel column is easily adapted for the determination of aflatoxins in corn and peanut meal. A combination of these techniques might be applicable to a variety of matrices.

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Experimental

Reagents

(a) *Mycotoxins.*—Standardize aflatoxins B_1, B_2, G_1, and G_2 in methanol as in **26.007** (4). Prepare dilutions in chloroform.

(b) *Internal standard.*—To correct for fluorescence losses observed with other matrices (2), develop aflatoxins on TLC plates with internal standard: an acetic acid ester of the —OH group in aflatoxin G_{2a}, prepared by the action of acetyl chloride on aflatoxin G_{2a}, with purification by preparative TLC (2).

(c) *Solvents.*—ACS grade in glass: formic acid, hexane (pesticide grade), iso-amyl alcohol, chloroform, methanol, acetone. For trace analysis, distill all solvents.

(d) *Oxalic acid-modified Florisil.*—To prepare large amounts, suspend 10.0 g Florisil (Fisher No. F-101) in 75 mL acetone and stir. Continue stirring for 10 min after addition of 4.85 g oxalic acid dihydrate in 30 mL acetone. Let suspension settle and remove supernatant liquid. Dry remaining solid overnight at 80°C, yielding 10.3 g. Deactivate with 7.5% (w/w) water and equilibrate 24 h on wrist-action shaker.

Apparatus

(a) *Styragel column.*—Prepare exclusion chromatographic system as in (1), except replace peristaltic pump by single piston pump (Analabs, Inc. Model A-60-S).

(b) *TLC plates.*—Purify TLC plates (Anasil O, TLC 099, Analabs, Inc., North Haven, CT) by letting them develop in methanol overnight. Remove from methanol, dry in warmed vacuum desiccator 1 h, and store in an atmosphere of 48% relative humidity (saturated KSCN solution).

(c) *Laser fluorescence systems.*—The system has been described in (2).

(d) *Switching valves.*—Each valve is 3-way slider-type (Altex) designed to work with standard Cheminert fittings and $^1/_{16}$ in. od Teflon tubing. Valves are mounted on a vertical bracket, above each other, immediately following the detector. All connections with other components (column, detector, syringe, etc.) use

Cheminert adapters and a minimum of 1/16 in. tubing.

Procedures

(a) *Extraction.*—For contaminated corn and peanut meal samples, extract 10 g in 55 mL chloroform–water (50 + 5) for 1 h, using wrist-action shaker. After filtering, remove solvent with rotary evaporator and dissolve residue in acetonitrile–chloroform (30 + 70). Filter through fine glass frit or 0.5 μm filter and dilute to 25 mL.

(b) *Operating parameters.*—For Styragel column, use mobile phase of acetonitrile-chloroform (30 + 70), pumped at 1.00 mL/min. Elution time of aflatoxins is determined by injection of standards. For 20 cm column, aflatoxins elute 11–14.5 min after injection. Develop TLC plates 1 h in formic acid–iso-amyl alcohol–hexane–chloroform (1.6 + 4 + 5 + 89.4).

Results and Discussion

Chemisorption by Florisil is probably due to formation of an organo-Mg complex. The likely points of interaction are the carbonyl groups on the aflatoxins, because acetone carbonyls disrupt the complex and elute the aflatoxins. An attempt was made to mask, or at least reduce, the extent of Mg interactions with the aflatoxins.

The purpose of the oxalic acid was to complex the most active Mg sites in the Florisil. Obviously, some Mg atoms will be more accessible to components in the mobile phase, while others will be previously complexed or occluded in the matrix (i.e., less active). The equimolar amount of oxalic acid added (3.8 mmol) would assure that all Mg atoms are complexed if necessary. However, it is unlikely that all the oxalic acid would be complexed, and some acid is recovered in the supernatant liquid.

This modified Florisil showed some residual chemisorptive properties and it was necessary to deactivate further with water. When 7.5% (w/w) deactivated adsorbent was packed into a 6 × 22 mm column, the recovery of all 4 aflatoxins was >90% for 4 replicates when 380 pg each pure standard was injected onto the column (92 ± 10% B_1, 94 ± 12% B_2, 91 ± 11% G_1, and 93 ± 11% G_2). The percent recovery was calculated by comparison with a sample of the same standard solution injected directly into a small flask. Both samples were then developed and analyzed on the same TLC plate. The recovery was best when aflatoxins were stripped from the Florisil column with 5 mL water–acetone (4 + 96).

In unmodified Florisil samples, aflatoxins were adsorbed at the top of the column. With Florisil samples that had been deactivated with oxalic acid and water, aflatoxins would elute slowly through the column while the fraction was being collected. Thus, while a 3.5 mL fraction was collected from the Styragel column, some aflatoxins would elute from a Florisil column less than 15 mm long. This extent of deactivation was considered necessary to prevent losses at low levels (<500 pg).

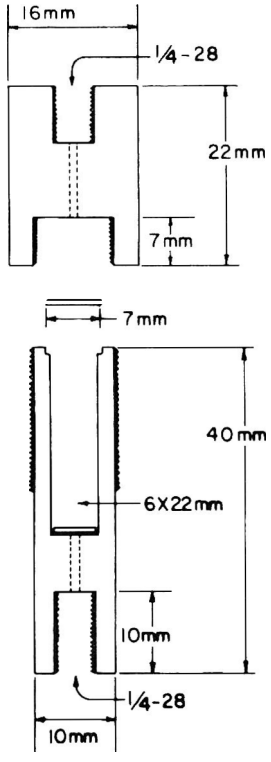

Figure 1. Schematic diagram of Florisil column.

It should be noted that aflatoxins elute from the Florisil in the order G_2, G_1, B_2, B_1. This is the order expected after elution from the Styragel column (i.e., reverse phase). Thus, losses due to early elution from the Florisil column are only of concern for G_2.

Figure 1 illustrates the design of the Florisil column. The 2 pieces are machined from a Teflon rod. The packing is held in place by 2 pieces of 270 mesh brass screen which have been soldered to small washers. The lower screen fits snugly into the packing compartment. The upper screen fits into a lip at the top of the packing compartment. A tight seal is made between the rim of the lower section and the upper cap. The external connections accept standard

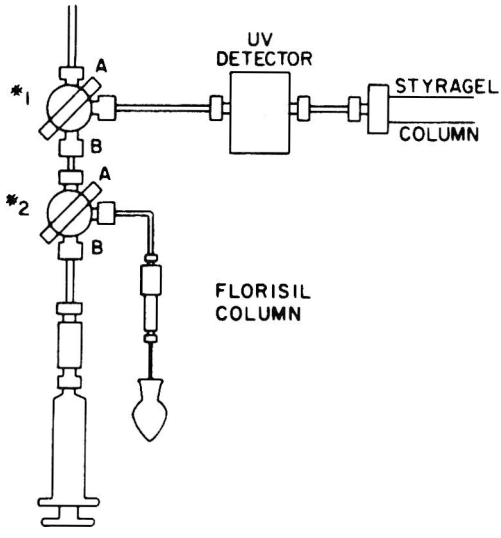

Figure 2. Schematic diagram of stream-switching valves between the Styragel and Florisil columns.

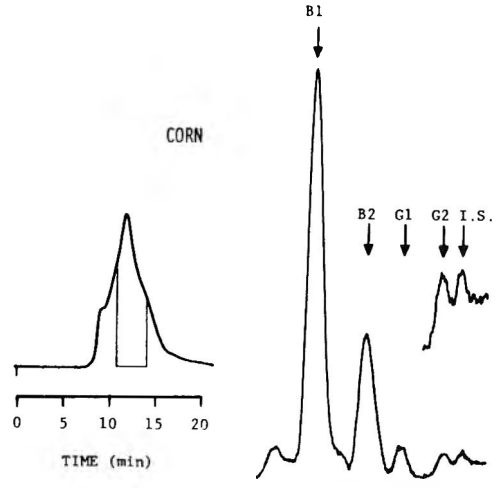

Figure 3. Contaminated corn sample: (a) Styragel elution profile, (b) TLC fluorescence scan showing aflatoxins present in sample. Internal standard is labeled I.S.

Cheminert fittings. Florisil is dry-packed into the column with tapping. Each column accepts approximately 300 mg Florisil.

Figure 2 illustrates the 2 valves used for stream switching between the Styragel and Florisil columns. When valve 1 is in position A, the mobile phase passes to a waste container. Position B diverts the mobile phase to valve 2, which must be in position A for collection of the aflatoxin-containing fraction. After the fraction has been collected, valve-1 is moved to position A and valve 2 can be moved to position B. This connects the Florisil column to a 5 mL syringe for elution of interfering components, if necessary, and then aflatoxins (5 mL water–acetone (4 + 96)).

Thus, while the remaining material is eluting from the Styragel column to a waste container, the sample is undergoing a second separation on the Florisil column. The aflatoxins are collected in a 10 mL pear-shape flask and the solvent is evaporated. Losses usually due to transfer of samples between columns are eliminated by the valves, and exposure to solvents is minimized without increasing the time required.

An example of the separating power of the Styragel column alone is shown in Figures 3 and 4. In the figures, the absorbance detector response is shown for injection of real samples onto the Styragel column. The bracketed area indicates the aflatoxin-containing fraction. The figures also show the resulting fluorescence scans obtained when the aflatoxin fractions were developed on TLC plates. For the corn sample, the aflatoxins and internal standard are clearly resolved from matrix components. For the peanut meal, one matrix component interferes with G_2, but the other aflatoxins are quantitated easily. The levels of aflatoxins in the corn sample range from more than 200 ppb for B_1 to 2.0 ppb for G_2. For the peanut meal, the levels range from 20 ppb B_1 to 3 ppb G_2.

Clearly, the Styragel column coupled with the Florisil column, if necessary, provides an effective cleanup of the sample matrix. These techniques should be applicable to a wide variety of sample matrices where aflatoxin contamination is of concern. It should also be emphasized that these procedures are useful for sample prepara-

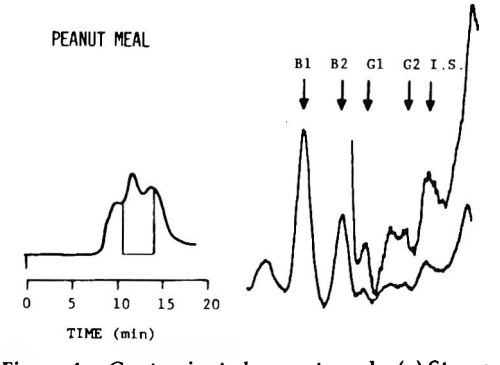

Figure 4. Contaminated peanut meal: (a) Styragel elution profile, (b) TLC fluorescence scan showing aflatoxins present in sample.

tion when liquid chromatography or other techniques are used for quantitation. In addition, with suitable precautions the Styragel column is re-usable for more than 6 months without serious deterioration.

Acknowledgment

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Gas-Liquid Chromatographic Determination of T-2 Toxin in Plasma

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A gas-liquid chromatographic method for the determination of T-2 toxin in plasma is described. The toxin is extracted with benzene, washed with aqueous sodium hydroxide, and chromatographed on a small Florisil column; the heptafluorobutyryl derivative is prepared by reaction with heptafluorobutyrylimidazole. The T-2 HFB derivative is chromatographed on OV-1 at 230°C and measured with an electron capture detector. Iso-T-2, an isomer of T-2 toxin, is added to samples as an internal standard before extraction. Recoveries averaged 98.0 ± 5.5% at levels ranging from 50 to 1000 ng/mL. The limit of detection is 25 ng/mL.

T-2 toxin, a 12,13-epoxytrichothecene mycotoxin, is a secondary metabolite produced by various species of *Fusarium* (1, 2). This compound has been implicated in sporadic but serious and often lethal cases of toxicoses in farm animals, including fusariotoxicoses in Canada (3) and moldy corn toxicoses in the midwestern United States (4).

Studies on the distribution and excretion of radiolabeled T-2 toxin have been conducted in swine (5), poultry (6, 7), and a lactating cow (8); however, at the present time, no pharmacokinetic experiments have been reported. Such experiments, however, require methods which are not only sensitive but also use only a minimal sample volume.

Methods currently available for analysis of T-2 toxin include procedures which use thin layer chromatography (9–11), flame ionization gas chromatography (9, 10), electron capture gas chromatography (10, 12), radioimmunoassay (13, 14), and gas chromatography–mass spectrometry (15, 16). The majority of methods, however, are not suitable for pharmacokinetic studies in farm animals because they are not sensitive enough, they are not adaptable to biological fluids, or they require sophisticated instruments not available in many laboratories. Although radioimmunoassay methods appear promising, the required antibodies and tritium-labeled T-2 toxin are not commercially available.

In the interest of developing a procedure for plasma analysis, we elected to use gas-liquid chromatography (GLC) with electron capture detection (ECD). The available GLC-ECD methods were originally developed for analysis of feeds and, although sensitive for these substrates, were not suitable for analysis of plasma without major modifications in extraction and cleanup.

The method presented here incorporates a modified heptafluorobutyryl derivatization technique and GLC-ECD steps reported by Romer et al. (12). The use of iso-T-2 as a true internal standard was also incorporated into the procedure, providing greater precision and reproducibility. Efficient and simple cleanup steps were then developed to complement the GLC-ECD final determination steps.

METHOD

Apparatus

(a) *Gas chromatograph.*—Hewlett-Packard Model 5840A equipped with 1.8 m × 2 mm id glass column packed with 3% OV-1 on 100–120 mesh Supelcoport, ^{63}Ni electron capture detector, and electronic integrator.

(b) *Tube shaker.*—Thermolyne Speci-Mix.

(c) *Chromatographic column.*—Polypropylene column with 30 mL reservoir (Supelco 5-8101, Bellefonte, PA 16823).

(d) *Test tubes.*—13 × 100 and 16 × 125 mm glass screw-cap tubes with Teflon-lined caps.

Reagents

(a) *Florisil.*—60–100 mesh (Fisher Scientific). Activate by heating 3 h at 120°C.

(b) *Heptafluorobutyrylimidazole (HFBI).*—Pierce Chemical Co., Rockford, IL 61105.

(c) *Sodium bicarbonate.*—5% w/v solution.

(d) *Potassium dihydrogen phosphate buffer.*—10% w/v solution.

(e) *Sodium hydroxide.*—0.05N NaOH with 4.0% KCl added.

(f) *Solvents.*—Benzene, hexane, chloroform, methanol, dichloromethane. All distilled in glass.

(g) *T-2 toxin.*—Mycolab Co., PO Box 321, Chesterfield, MO 63107 (Figure 1).

(h) *Iso-T-2.*—Synthesize from HT-2 as described (17). T-2 is hydrolyzed to HT-2 with

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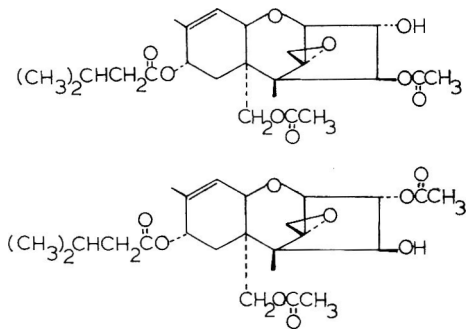

Figure 1. Chemical structures of T-2 toxin (top) and iso-T-2 toxin (bottom).

ammonium hydroxide in methanol. HT-2 is partially acetylated with acetic anhydride and pyridine to yield iso-T-2. Prepare working standard containing 25 μg/mL ethanol.

Sample Preparation

Centrifuge all blood samples immediately after collection and store plasma at −20°C until day of analysis. Thaw and mix samples thoroughly before extraction.

Extraction and Cleanup

Add 2.5 mL plasma to 13 × 100 mm test tube containing 0.5 mL phosphate buffer and 50 μL iso-T-2 internal standard. (If amount of T-2 detected is beyond linear range, re-extract smaller aliquots of plasma and dilute with water to give final volume of 2.5 mL.) Transfer 4.0 mL benzene to tube, cap, and mix 10 min on tube shaker. Centrifuge 5 min at 2000 rpm. Pipet benzene extract into clean 16 × 125 mm test tube. Repeat extraction of plasma with additional 4 mL benzene and combine extracts. Add 2 mL NaOH to benzene extracts and mix 1 min on tube shaker. Transfer benzene layer to clean test tube. Repartition base with additional 2 mL benzene, combine benzene extracts, and add 4 mL hexane.

Florisil Chromatography

Prepare Florisil column as follows: Tamp small ball of glass wool at bottom of column and add, in order, 1 cm anhydrous sodium sulfate, 1 g Florisil, and 1 cm anhydrous sodium sulfate. Columns can be prepared in advance and stored at least 1 week in desiccator.

Prewash column immediately before use with 10 mL chloroform–methanol (95 + 5) followed by 10 mL hexane. Do not let column run dry at any time. When hexane is 1 cm above sodium sulfate, add sample extract. Wash column with 10 mL dichloromethane of which 2 mL is used to rinse the test tube. Elute T-2 with 15 mL chloroform–methanol (95 + 5) into 16 × 125 mm tube. Concentrate to dryness under stream of nitrogen and gentle heat ca <50°C.

Derivatization

Redissolve residue in 1 mL toluene. Add 50 μL HFBI, cap, and mix 30 s. Add 1 mL bicarbonate and mix until toluene layer is clear. Add 4.0 mL hexane, mix, and centrifuge 5 min at 2000 rpm.

Gas Chromatography

Inject 4 μL organic layer into gas chromatograph, using following conditions: column 230°C; injector 250°C; detector 325°C; argon–methane (95 + 5) carrier gas flow 22 mL/min. Inject standard and blank samples daily. With procedure described above, 2.0 μL equivalents of plasma are injected into gas chromatograph. Retention times are 8.60 and 9.49 min for T-2 and iso-T-2 toxin, respectively.

Results and Discussion

Initial studies used ethyl ether and ethyl acetate as extraction solvents for partitioning T-2 toxin from plasma. Although extraction efficiencies of 85–90% were observed, a large amount of impurities was also co-extracted. The use of benzene as an extraction solvent provided efficiencies similar to that obtained with ethyl ether; however, the resulting extract contained fewer interferences.

Base partitioning has previously been used as a cleanup step before determination of T-2 toxin and diacetoxyscirpenol in animal feeds (12). The base wash was simple and rapid, and efficiently removed acidic components present in the plasma extracts. Because impurities were detected in the base solution, the sodium hydroxide was partitioned once with equal volumes of benzene before use. No loss of either T-2 toxin or the internal standard iso-T-2 was observed at this step.

The Florisil column was reduced in size to minimize the solvents required, the time involved, and the total cost of analyses. One g Florisil was sufficient to provide the necessary cleanup of sample extracts. The polypropylene columns were inexpensive enough to be disposable, yet adequately durable so they could be washed and reused if desired. Polarity and volume of the elution solvent were adjusted to provide close to 100% recovery from the column

Table 1. Recovery of T-2 toxin added to plasma

Amt added, ng/mL	N	Recovery, % (mean ± SD)
50	5	100.5 ± 9.1
100	6	100.1 ± 5.3
250	7	97.0 ± 2.9
500	4	96.3 ± 9.8
1000	7	96.4 ± 3.5
Total	29	98.0 ± 5.5

in a fraction (15 mL) collected directly into a test tube suitable for derivatization. This decreased the time of analysis by eliminating one transfer step. The Florisil columns could be prepared in advance and stored in a desiccator for at least one week without affecting elution profiles.

Preliminary work used benzene as the derivatizing solvent; however, toluene worked equally well and was substituted in later analyses. Derivatization was quantitative with 50 μL HFBI.

The electron capture detector is very sensitive to the heptafluorobutyrate of T-2 toxin. Romer et al. (12) described a minimum sensitivity of 150 pg T-2 toxin, using an electron capture detector. By using a higher column temperature and diluting the sample extract less than previously reported, 25 pg standard T-2 could be detected. In actual plasma samples, however, 50 pg was required for identification.

Detector response to T-2 heptafluorobutyrate was linear up to 2.0 ng injected, using peak areas determined by electronic integration. Detector response varies slightly from day to day, so standards must be injected daily. Using the method as described, up to 2.0 μL equivalents of plasma are injected into the gas chromatograph, resulting in a detection limit of 25 ng/mL.

Initial recovery experiments were performed by adding T-2 toxin to plasma and quantitating by using an external standard. Using this method, absolute recoveries of T-2 toxin averaged 83.6% over a range of 100–1000 ng/mL, and recoveries of the internal standard iso-T-2 averaged 85.2%. By adding iso-T-2 directly to the plasma before extraction and using the internal standard method of quantitation, recoveries of T-2 toxin (normalized to the recovery of the internal standard iso-T-2) averaged 98.0% (Table 1). The coefficient of variation was also good (5.5%) with the use of this internal standard for quantitation. Because iso-T-2 and T-2 toxin are isomers, their chemical properties such as extraction efficiency and Florisil column elution profiles are nearly identical. The 2 compounds, however, still separated sufficiently under the described gas chromatographic conditions for accurate quantitation. Because of similar chemical behavior between these trichothecenes, small variations in recovery from sample to sample did not reduce the precision of the assay, as indicated by the low coefficient of variation.

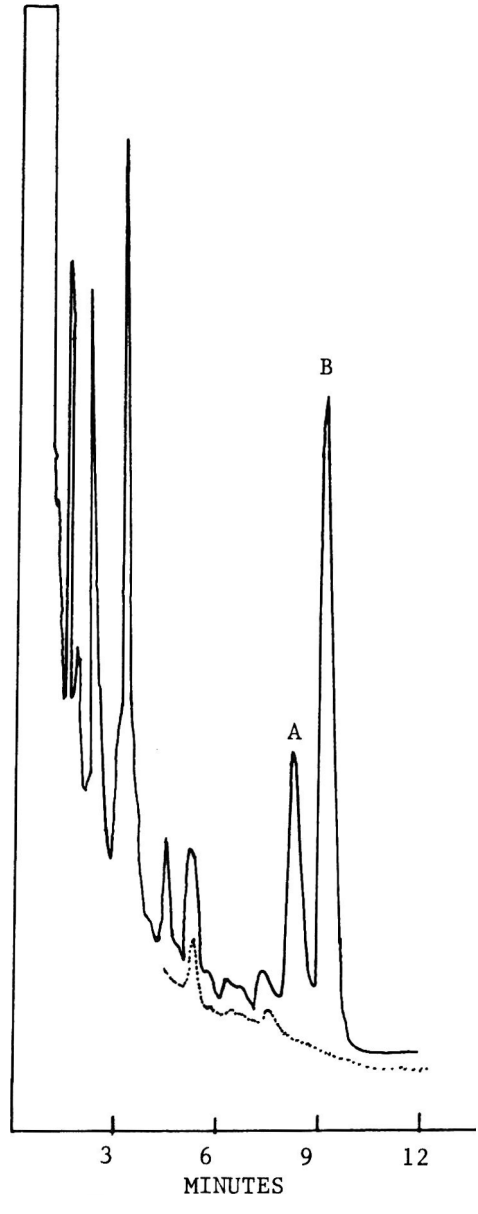

Figure 2. Gas chromatogram of plasma extract. Dotted line represents control; solid line plasma spiked with 250 ng/mL T-2 toxin (A) and internal standard iso-T-2 (B).

The gas chromatographic portion of the anal-

ysis could be completed within 12 min per sample. Higher column temperatures could be used to reduce the analysis time; however, at higher temperatures T-2 toxin did not separate as well from the iso-T-2 internal standard. A column temperature of 230°C provided the best compromise between resolution of T-2 and iso-T-2, and the total time required for GC analysis.

Extracts of plasma were colorless, and control samples produced chromatograms without interferences at the retention time of either T-2 or iso-T-2 (Figure 2). In addition, no late-eluting peaks were observed which could interfere with subsequent injections.

Two variables were critical to obtain chromatograms free from interfering peaks. It was necessary that plasma samples be clean and obtained from unhemolyzed blood. Compounds present in hemolyzed blood produced peaks which eluted just prior to T-2 toxin. If the blood was sufficiently hemolyzed, the interfering peak was large enough to interfere with quantitation. The derivatizing agent was also very important. Heptafluorobutyrylimidazole which was old or which contained a pinkish color did not actually interfere with derivatization, although it did provide extraneous peaks on the chromatograms which sometimes affected quantitation.

The method described was designed for analysis of blood samples for investigations on the toxicokinetics and of T-2 toxin in swine and cattle. Such studies require a sensitive analytical method which can be used with relatively small sample sizes. The method developed fulfilled the above requirements. Studies on the toxicokinetics and toxicodynamics of T-2 toxin in swine and cattle are currently in progress and will be described in a subsequent paper.

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Rapid High Pressure Liquid Chromatographic Determination of Aflatoxin M_1 in Milk and Nonfat Dry Milk

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A modification of the current revised AOAC method, 26.A10-26.A15, is described for the rapid analysis of aflatoxin M_1 in milk and nonfat dry milk. The method incorporates chloroform extraction and eliminates the need for column chromatography by using liquid-liquid partition for sample extract cleanup. Quantitation is carried out by using fluorescence detection combined with high pressure liquid chromatography (HPLC) of aflatoxin M_1 which has been converted to aflatoxin M_{2a} with trifluoroacetic acid. The method has a detection limit of 0.014 μg/L (2 × signal/noise) for whole milk. For 6 samples of naturally contaminated nonfat dry and freeze-dried milk, the modified method gave an average result of 0.698 μg/L; the AOAC method gave an average result of 0.386 μg/L.

The current AOAC method (1, 2) specifies chloroform as the extracting solvent and thin layer chromatography (TLC) for the detection and quantitation of aflatoxin M_1. In our laboratories, chloroform was found to extract aflatoxin M_1 efficiently; however, when present in milk at levels less than 0.5 μg/L, M_1 is difficult to detect by the AOAC method because of interfering fluorescent materials. Radioimmunoassay and enzyme-linked immunosorbent assays have recently been introduced (3), and have detection limits of 0.24 μg/L in artificially contaminated milk. Other methods involving column chromatography (4-6) and using TLC for detection and quantitation have been published. Knutti et al. (7) and Beebe and Takahashi (8) first used derivatization and fluorescence detection by high pressure liquid chromatography (HPLC) for determination of M_1 in milk products. Recently, an HPLC method was introduced using Sep-Pak cartridges for cleanup and fluorescence detection of M_1 in dairy products (9); the detection limit (0.1 ppb) is comparable to that for TLC. The method developed in our laboratories and described here uses a straightforward chloroform extraction, liquid-liquid partition cleanup and derivatization of M_1 to M_{2a} using trifluoroacetic acid, followed by HPLC quantitation using a fluorescence detector to achieve a detection limit of 0.014 μg/L.

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METHOD

Reagents

(a) *Mobile phase.*—Combine 250 mL acetonitrile, 750 mL water, and 10 mL glacial acetic acid, and add 1.0 g anhydrous sodium acetate. Stir slowly and continually during use. Ratio of acetonitrile and water can be varied as necessary to adjust retention time and resolution.

(b) *Saturated salt solution.*—Place 400 g sodium chloride into 2 L bottle containing 1 L water. Warm gently to accelerate solution. Some salt should remain undissolved. Cool to room temperature before using.

(c) *Injection solvent.*—Combine 100 mL acetonitrile, 900 mL water, and 20 mL glacial acetic acid. Shake or stir before use.

(d) *Standard stock solution.*—0.20 μg/mL. Dissolve 40 μg (as received) aflatoxin M_1 standard (Eureka Labs, Sacramento, CA 95816) in 200 mL chloroform. Store in freezer. Solution is stable 2 months.

(e) *Working standards.*—High standard 0.0148 ng/μL; low standard 0.0049 ng/μL. Pipet 300 and 100 μL portions of stock solution into separate 2 dram vials. Evaporate to dryness under stream of nitrogen with gentle warming and add 50 μL trifluoroacetic acid (TFA). Stir with vortex mixer, wait *at least* 15 min, add 4.00 mL injection solvent, and stir again with vortex mixer. Store in freezer until ready to use. Solution is stable only 1 week. Bring to room temperature protected from light just before injection.

Apparatus

(a) *HPLC pump.*—Model 110 constant flow (Altex, Berkeley, CA 94710), or equivalent.

(b) *HPLC injector.*—Model LC420 Autosampler equipped with 20 μL loop (Perkin Elmer, Norwalk, CT 08856), or equivalent.

(c) *HPLC detector.*—Model 650-10LC fluorescence spectrophotometer (Perkin Elmer), or equivalent. Set excitation for 365 nm with 20 nm slit width. Set emission for 440 nm with 20 nm slit width.

(d) *HPLC column.*—Du Pont Zorbax C8 4.6 mm × 25 cm (Du Pont, Wilmington, DE 19898), or

reverse phase C8 5 mm radial compression column fitted in RCM 100 compression module (Waters Associates, Milford, MA 01757), or equivalent.

Procedure

(1) Pipet 50 mL fluid milk into 250 mL separatory funnel, and add 10 mL saturated salt solution and 120 mL chloroform (prewarmed to ca 35°C). Proceed to step 3.

(2) For powdered or freeze-dried milk, reconstitute 5.0 g with 50 mL water by shaking in 250 mL separatory funnel. Add 10 mL saturated salt solution and 120 mL chloroform (prewarmed to ca 35°C) and proceed to step 3.

(3) Roll mixture very gently for 3 min. Let layers separate. (There should be no emulsion formation if care is taken in 3-min rolling. If emulsion forms, centrifuge at 14 500 rpm for 10 min, pour carefully back into separatory funnel, and let layers separate.) Drain lower chloroform layer into 250 mL Erlenmeyer flask. Add 10 g anhydrous sodium sulfate to chloroform extract. Stir chloroform solution occasionally for 3 min and filter through fluted No. 44 paper into 100 mL graduate. Record volume collected.

(4) Evaporate chloroform extract to dryness under vacuum with gentle heating (60°C). Immediately transfer residue to 250 mL separatory funnel, using 50 mL (divided in 3 portions) of acetonitrile. Extract acetonitrile solution with two 50 mL portions of petroleum ether. Discard petroleum ether and transfer acetonitrile to 250 mL round-bottom flask. Evaporate acetonitrile solution to dryness.

(5) Immediately transfer residue to 2 dram vial with minimal amount of methylene chloride. Evaporate to dryness under stream of nitrogen with gentle heating, add 50 μL TFA, mix with vortex mixer for 20 s, and wait *at least* 15 min. Add, by pipet, 4.0 or 2.0 mL (for samples of low M_1 concentration) of injection solvent. Remix on vortex mixer.

HPLC Quantitation

Set flow rate at 1.5 mL/min for Zorbax C8 or 3.0 mL/min for Waters RCM C8, and let column equilibrate 30–45 min. Repeat injections of standard solutions until peak heights are constant. Inject sample extracts interspersed with standards as frequently as necessary to ensure accurate quantitation.

Calculations

For fluid milk:

$$\text{Aflatoxin } M_1, \mu g/L = C \times PH_{sam} \times (V_1 + 50)/(PH_{std} \times V_2)$$

where V_2 = original sample volume (mL) × filtrate volume/120; C = concentration of M_1 std, ng/μL; PH_{sam} = sample peak height; PH_{std} = standard peak height; V_1 = μL injection solvent used to dissolve sample (usually 4000 or 2000).

For dry milk:

$$\text{Aflatoxin } M_1, \mu g/kg = C \times PH_{sam} \times (V_1 + 50)/(PH_{std} \times W)$$

where W = original weight, g × filtrate volume/120.

Results and Discussion

Shaking the aqueous milk solution with chloroform as specified in the current AOAC method invariably causes emulsion formation and reduces the volume of chloroform extract recovered. Rolling the separatory funnel very gently for 3 min instead of shaking for 1 min successfully eliminates emulsion formation. Extraction efficiency is retained as evidenced by the high recovery results.

The effective removal of most of the fluorescent interferences by the petroleum ether–acetonitrile partition from the sample and the enhanced fluorescence sensitivity gained by the trifluoroacetic acid conversion of M_1 to M_{2a} are illustrated in Figure 1, where the peak representing 0.07 μg/L of M_1 in a milk sample is easily measurable and free of interfering peaks. Figure 2 shows a chromatogram of derivatized M_1 standard (0.0049 ng/μL); Figure 3 shows a chromatogram of an un-derivatized M_1 standard (0.0049 ng/μL). It is evident that the sensitivity of the M_1 analysis is enhanced more than 3-fold by converting M_1 to M_{2a}. Based on the signal-to-noise level for the M_{2a} peak in Figure 1, the detection limit for aflatoxin M_1 at 2× noise level would be 0.014 μg/L in the original sample. Figure 4 is a superimposition of Figure 3 on Figure 2, showing the complete conversion of M_1 to M_{2a} by the lack of detector response at 9.5 min. Figure 5 shows chromatograms of (a) derivatized M_1 standard, and (b) derivatized M_1 in a naturally contaminated milk sample representing 0.05 μg M_1/L in original sample.

Six samples of artificially contaminated milk (spiked by adding a measured quantity of aflatoxin M_1 into a fixed volume of milk at room temperature), one sample of naturally contaminated nonfat dry milk, and 3 samples of naturally contaminated freeze-dried milk (milk obtained from cows fed aflatoxin-positive feed) were se-

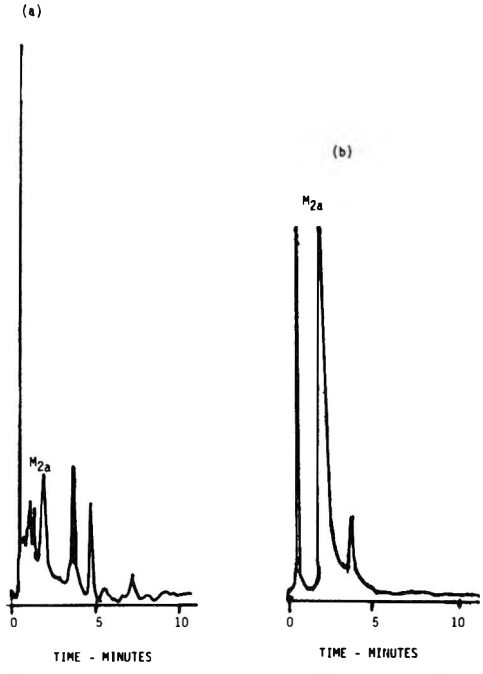

Figure 1. Chromatograms of (a) M_{2a} (representing 0.07 μg M_1/L in original sample) from artificially contaminated milk sample, and (b) M_{2a} from derivatized M_1 standard (0.098 ng or 0.0049 ng M_1/μL). Waters RCM100 C8 5 mm column with acetonitrile-water-acetic acid (250 + 750 + 10) + 0.1% anhydrous sodium acetate at 3.0 mL/min, attenuation at 3.

lected for this study. Each sample was analyzed in duplicate both by this method and by a revised AOAC method which used the extraction and cleanup described in **26.A12** and **26.A13.** After column chromatography (**26.A13**), the samples were derivatized and quantitated as described in step 5 of the procedure above. The results are shown in Tables 1 and 2. The relative standard deviation by the present method was 14.1% and by the AOAC method 22.9%. For artificially contaminated samples, the AOAC extraction method gave an average recovery of 66.7% ± 8.94 (13.4% rel. SD) and the short method gave an average recovery of 100.2% ± 13.5 (13.4% rel. SD). For naturally contaminated samples, the short method provides average results 1.86 times as high as average results obtained by the AOAC method. Elimination of the column chromatography cleanup step necessary in the AOAC method may reduce M_1 loss during workup.

Results of this study indicate that this short method, coupled with fluorescence detection of the derivatized M_1, provides improved extraction efficiency, improved sensitivity, and in our ex-

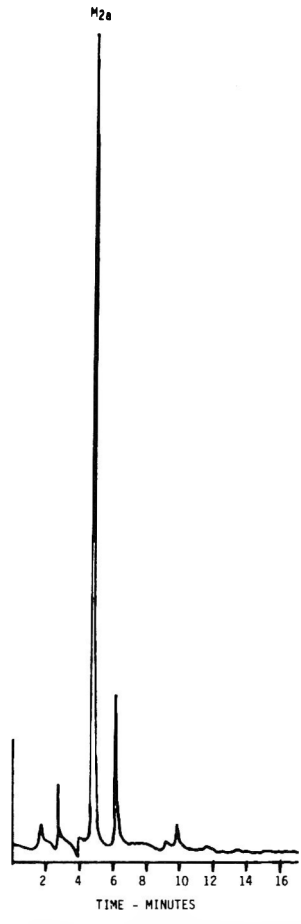

Figure 2. Chromatogram of M_{2a} from derivatized M_1 standard (0.098 or 0.0049 ng M_1/μL). DuPont Zorbax C8 4.6 mm id × 25 cm with acetonitrile-water-acetic acid (30 + 70 + 10) + 0.1% anhydrous sodium acetate at 1.0 mL/min, attenuation at 3.

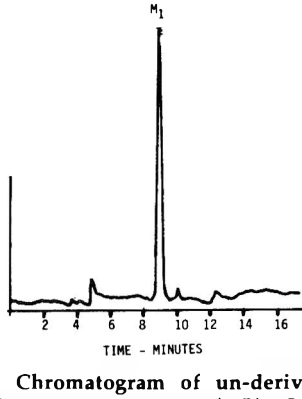

Figure 3. Chromatogram of un-derivatized M_1 standard. (0.098 or 0.0049 ng M_1/mL). Same conditions as in Figure 2.

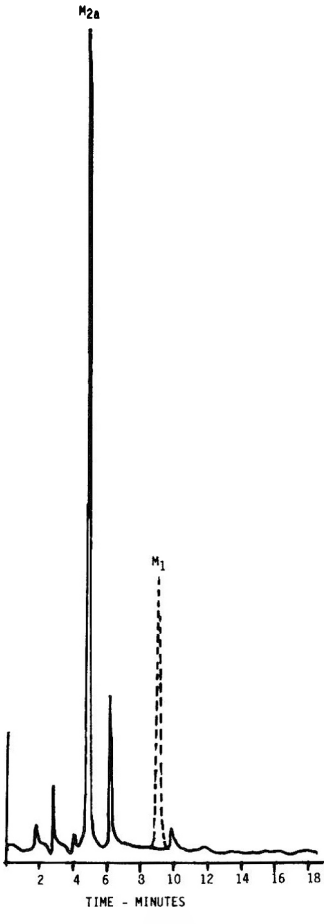

Figure 4. Superimposition of Figure 3 on Figure 2 showing the respective retention times of M_1 and M_{2a}. Note complete conversion of M_1 to M_{2a} shown by lack of detector response at 9.5 min. Conditions as in Figure 2.

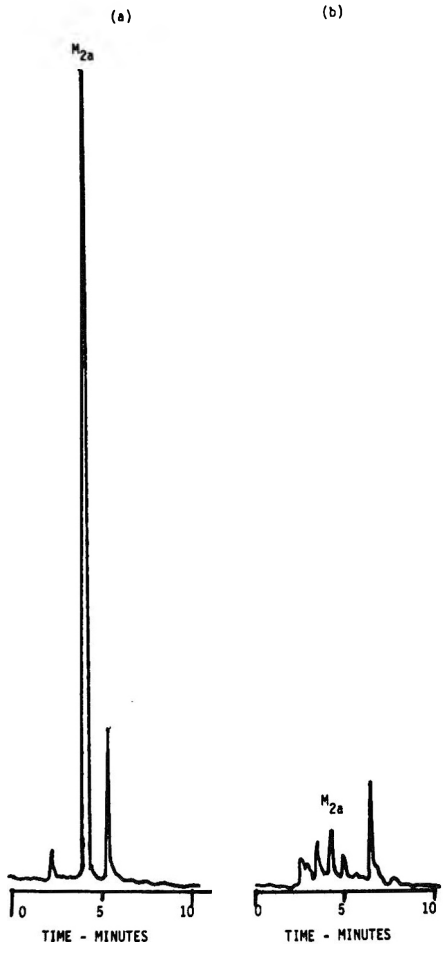

Figure 5. Chromatograms of (a) M_{2a} from derivatized M_1 standard (0.0296 or 0.0148 ng $M_1/\mu L$). (b) M_{2a} (representing 0.05 μg M_1/L in original sample) from naturally contaminated milk sample. Conditions as in Figure 2.

Table 1. Recovery of aflatoxin M_1 ($\mu g/L$) from artificially contaminated milk samples

Sample	Added	AOAC method	This method
9a	0.1	0.07	0.11
9b	0.1	0.08	0.11
10a	0.2	0.14	0.24
10b	0.2	0.14	0.22
1a	0.5	0.32	0.47
1b	0.5	0.40	0.57
2a	1.0	0.64	0.96
2b	1.0	0.72	0.80
3a	4.0	2.24	4.14
3b	4.0	2.56	3.95
4a	8.0	3.96	6.19
4b	8.0	4.88	7.11
Av. rec. (%) ± SD		66.7 ± 8.94	100.2 ± 13.5
Rel. SD, %		13.4	13.4

Table 2. Determination of aflatoxin M_1 ($\mu g/L$ as reconstituted) in naturally contaminated freeze-dried milk

Sample	AOAC method	Av.	This method	Av.
5a	0.24	0.245	0.40	0.40
5b	0.25		0.40	
6a	0.506	0.503	0.987	0.963
6b	0.500		0.939	
7a	0.20	0.17	0.37	0.38
7b	0.14		0.39	
8a	0.60	0.625	1.06	1.05
8b	0.65		1.04	
Av.		0.386		0.698
Rel. SD, %		22.9		14.1
Av. this method/ av. AOAC method			1.81	

perience requires substantially less time than current TLC and HPLC methods.

Acknowledgments

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METALS AND OTHER ELEMENTS

Critical Re-appraisal of Fluorometric Method for Determination of Selenium in Biological Materials

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The reaction of 2,3-diaminonaphthalene (DAN) with Se(IV) to form a fluorescent Se-DAN complex is the basis of a fluorometric method for determination of Se. With the aid of metabolically incorporated ^{75}Se the method was critically re-examined. The study showed that loss of ^{75}Se was negligible when liver or blood was microwave-dried or thermally dried at temperatures up to 120°C; during HCl reduction of Se(VI) to Se(IV), a temperature up to 210°C could be used with no loss of ^{75}Se; it was unnecessary to perform pH adjustment of solution for formation of Se-DAN complex before solvent extraction; it was unnecessary to carry out the chelation/extraction step in diffuse light; solvent phase containing Se-DAN complex could be left in contact with aqueous phase up to one week under fluorescent light with no effect on analytical results. From this study we were able to dispute or overcome a number of criticisms and myths which have been laid against the fluorometric method for many years. As a result, an improved single tube method was developed. The within-batch variation of the improved method was about 2%, while the between-batch variation over a period of 2 years was less than 10%. The method can handle 200 samples per batch and is applicable to a wide range of biological samples including liver, orchard leaves, barley, wheat, lucerne (alfalfa), poultry feed, fish, hair, blood, urine, and milk.

Of several methods available for the determination of selenium (Se) in biological materials, fluorometry and hydride generation atomic absorption spectrophotometry are the more commonly used. In a recent survey (1), it was shown that 68% of the participants reported using the fluorometric method and 13% used the hydride generation method.

The fluorometric method involves the reaction of 2,3-diaminonaphthalene (DAN) with Se(IV) to form a fluorescent Se-DAN piazselenol. This reaction was first described by Parker and Harvey in 1962 (2); the kinetics of the reaction were studied by Cukor and Lott (3). Many reports have since appeared on applying the reaction to determination of Se (4–8). The methods are generally tedious and capable of handling relatively small numbers of samples per batch of analyses; the digestion procedure requires constant attention; there are multiple transfers of solutions, and bulky glassware such as Kjeldahl flasks and separating funnels are required; adjustment of pH before solvent extraction was thought necessary and the analytical procedures were carried out in diffuse light. Consequently, several reports have appeared on improving the methods. Chan (9) proposed using a 15 × 150 mm test tube in place of the 100 mL Kjeldahl flask for digestion. Whetter and Ullrey (10) substituted the Kjeldahl flask with Erlenmyer flasks and carried out all reactions in the same flask. Both methods specified a temperature-controlled block for acid digestion of samples. Spallholz et al. (11) used a 19 × 150 mm test tube and acid-digested the samples on a Fisher burner. Michie et al. (12) described the use of a mixture of HNO_3, $HClO_4$, and H_2SO_4 for digestion and glycerin/HCl buffer for pH adjustment. Brown and Watkinson (13) and Szydlowski and Dunmire (14) used an AutoAnalyzer to perform the fluorometric assay. In spite of the analysts' efforts to modify the fluorometric method, an improved method which does not contain any of the weaknesses mentioned earlier has not been reported. This paper details our re-appraisal of the published methods and describes a modified fluorometric method for determination of Se in a wide range of biological materials.

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METHOD

Apparatus

(a) *Culture tubes.*—Pyrex, 20 × 150 mm (Corning Cat. No. 9828/20X).

(b) *Teflon-lined screw cap.*—Yellow type (Schott-Jena Cat. No. 292401).

(c) *Hotplates.*—2400 W (Labmaster, Drug House of Australia Ltd) modified to have temperature- and time-controlled mechanisms.

(d) *Aluminum blocks.*—285 × 285 × 80 mm. Cast at a local aluminum foundry. Each block has 121 holes (22 mm diam.) drilled at 25 mm centers and at 62 mm depth to accept culture tubes. Five holes of same depth but of 7 mm diam. were interspersed among tube holes to accommodate thermometer (0–360°C).

(e) *Aluminum draft shield.*—305 × 305 × 200 mm. Custom-made to fit over aluminum block to facilitate acid digestion.

(f) *Wire racks.*—Plastic-coated, capable of accommodating 100 culture tubes each.

(g) *Mechanical mixer.*—Hand-operated (laboratory-made) with lid to accept wire rack (f).

(h) *Stainless steel tongs.*—200 mm long (Gallenkamp, London, UK. Cat. No. TNS-4IOU). Modified by bending region between pivot and tips at right angles. Clear plastic tubing fitted over tips.

(i) *Glass beads.*—1 mm, acid-washed, (Selbys Scientific Ltd, Australia).

(j) *Separatory funnels.*—250 mL. Fitted with Teflon taps.

(k) *Pipets.*—Dispensette (0–5 mL) and Oxford Pipettor (0–1 mL and 0–10 mL).

(l) *Microwave oven.*—Rank Litton Minutemaster Model 7425-100, with microwave frequency of 2450 MHz and power output of 650 W.

(m) *Spectrofluorimeter.*—Perkin Elmer 1000 M fitted with 600 μL microflow cell in conjunction with Coleman 055-0200 Autosampler and interfaced with Hewlett-Packard 97S i/O calculator.

(n) *Gamma crystal counter.*—LKB Wallac 80000 with following settings for ^{75}Se counting: channel width, 60 V; discrimination level, 250 V; high voltage 495 V.

(o) *pH meter.*—Radiometer 28 with calomel-glass electrode system.

Reagents

(a) *Nitric acid.*—16M analytical grade (Univar, Ajax Chemicals, Sydney, Australia).

(b) *Perchloric acid.*—11.6M analytical grade (Univar, Ajax Chemicals).

(c) *Hydrochloric acid.*—11.3M: Analar, BDH Chemicals (Australia) Pty Ltd. 0.1M: Dilute 8.9 mL 11.3M HCl to 1 L with water.

(d) *Acid mixture.*—Mix 16M HNO_3 and 11.6M $HClO_4$ (4 + 1 v/v).

(e) *Cyclohexane.*—Spectroscopic grade, Merck Cat. No. 2822.

(f) *Disodium ethylenediaminetetraacetic acid (Na_2H_2EDTA) solution.*—0.1M: Dissolve 32.2g $Na_2H_2EDTA{\cdot}2H_2O$ (analytical grade, Ajax Chemicals) and dilute to 1 L with glass-distilled water. 0.04M: Dilute 2 volumes of 0.1M Na_2H_2EDTA with 5 volumes distilled water. 0.0025M: Dilute 50 mL 0.1M Na_2H_2EDTA solution to 2 L with glass-distilled water.

(g) *2,3-Diaminonaphthalene (DAN) solution.*—0.1%. Weigh 0.1 g DAN (97% purity, Aldrich Chemical Co., Milwaukee, WI 53233. Cat. No. 13653-0) into 250 mL conical flask. Add 100 mL 0.1M HCl and place flask in 60°C water bath. Agitate flask occasionally to help dissolution of DAN. About 10 min is required for complete dissolution. Cool flask under running water, and transfer DAN solution to separatory funnel fitted with Teflon tap. To separatory funnel, add 25 mL cyclohexane per 100 mL DAN solution and shake vigorously for 30 s. Let phases separate. Drain aqueous phase (DAN) into clean separatory funnel and repeat washing procedure 3 more times, discarding cyclohexane layer each time. Prepare fresh DAN solution for each batch of analyses.

(h) *EDTA-DAN solution.*—Mix 0.04M EDTA with 0.1% DAN (1 + 1 v/v). Solution must be dispensed into culture tubes within 15 min of preparation, otherwise EDTA may crystallize.

(i) *Selenium standard solutions.*—*Stock solution:* 100 μmol Se/L. Dissolve 0.01729 g sodium selenite (Na_2SeO_3) in 1 L 0.1M HCl. *Working standards:* 0.25, 0.50, 1.00, and 2.00 μmol Se/L. Dispense 5 mL stock standard into 2 L, 1 L, 500 mL, and 250 mL volumetric flasks, respectively, and dilute to volume with 0.1M HCl. Retain 1 L 0.1M HCl used to prepare standards as standard blank. Also prepare 5 μmol Se/L standard by diluting 5 mL stock standard to 100 mL with 0.1M HCl.

(j) *L-Selenomethionine.*—Injection BP, 100 μCi ^{75}Se/mL (Radiochemical Centre Ltd, Amersham, UK).

Glassware Decontamination

All glassware was washed in detergent (Pyroneg, Diversey (A. Asia) Pty Ltd), soaked overnight in 1.6M HNO_3, and rinsed several times with glass-distilled water before drying in 100°C cabinet.

Experimental

Pyrex culture tubes (20 × 150 mm) were used throughout the investigation. In addition to use of fluorescence response to evaluate analytical conditions, radioactive Se as L-selenomethionine (^{75}Se) was used. Liver and blood samples containing metabolically incorporated ^{75}Se were obtained from a rabbit (2 kg body weight) 48 h

after the animal had received an intraperitoneal injection of 100 μCi ^{75}Se. An LKB Wallac gamma crystal counter was used to monitor ^{75}Se radioactivity.

The following factors were examined before establishing the improved method: (a) effect of drying temperature on loss of ^{75}Se from liver and blood; (b) suitability of HNO_3–$HClO_4$ acid mixture in different proportions for digesting samples; (c) effects of temperature and different volumes of concentrated HCl in reducing Se(VI) to Se(IV); (d) suitability and quantity of glass-distilled water as diluent for digests; (e) effect of pH on Se-DAN complex formation; (f) feasibility of combining EDTA and DAN solutions to allow 1-step addition of these reagents; (g) addition of cyclohexane to reaction mixture before incubation in 60°C water bath; (h) length of incubation time for Se-DAN complex formation; (i) stability of Se-DAN complex with time.

All investigations were performed in quadruplicate unless indicated otherwise.

Procedure

Digestion.—Place several anti-bumping glass beads into 20 × 150 mm culture tubes. Add known volume/weight of samples to culture tubes (suggested quantities: 1–2 mL for blood, milk, and urine; 2–4 mL for plasma or serum (haemolysis-free); 0.1–0.5 g (dry weight) for liver, kidney cortex, wheat, barley, orchard leaves, other plant materials, hair, or fish; 0.5–1.0 g for egg homogenate). If quantities greater than those suggested are used, charring may occur and result in loss of volatile selenium. Take care when digesting fatty samples which may react violently with perchloric acid. As a guide, 0.10 g macadamia nut containing ca 78% crude fat can be digested without charring.

Set up 6 tubes for selenium standards: 0, 0.25, 0.50, 1.00, 2.00, and 5.00 μmol/L. Add 1 mL of each standard to respective culture tubes. Dispense 4 mL acid mixture with Brand Dispensette (0–5 mL) to all tubes and let stand at least 1–2 h. Place tubes in aluminum block and set temperature to reach 210°C. Place aluminum draft shield over block to facilitate digestion. Heat to fumes of $HClO_4$. If heating is to be commenced in the evening, tubes can be left heating overnight and digests should be clear with $HClO_4$ fuming by next morning. Remove aluminum shield and take tubes from aluminum block by using the modified tongs. Cool tubes and add 0.5 mL 35% HCl from an Oxford Pipettor (0–1 mL). Heat tubes 30 min in aluminum block at 100–150°C to reduce Se(VI) to Se(IV). Cool tubes and add 16 mL 0.0025M Na_2H_2EDTA solution.

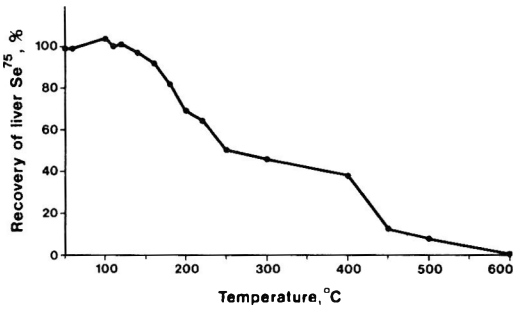

Figure 1. Effect of drying temperature on loss of metabolically incorporated ^{75}Se from rabbit liver. Each point represents the mean of 4 determinations.

Solvent extraction.—The following operation can be performed under laboratory fluorescent light. Add 1 mL DAN solution followed by 5 mL cyclohexane. Tightly screw Teflon-lined caps onto culture tubes. Place wire rack containing culture tubes in mechanical mixer and mix contents of each tube by alternately turning rack clockwise and counter-clockwise several times. Place rack in 60°C water bath for 30 min. Remove rack from bath, place rack in mechanical mixer, and solvent-extract Se-DAN complex for 1 min. Let tubes stand 5 min to allow phase separation. Set fluorometer at excitation wavelength 364 nm, emission wavelength 523 nm, emission slit width W; scale expansion 10×, sampling volume 2 mL, and integration time for read mode 5 s. Calculate results manually, or interface fluorometer with calculator and have all results computed by means of a program.

Results

Effect of Drying Temperature on Se Loss

When blood containing metabolically incorporated ^{75}Se was dried for 48 h, the mean and standard deviation for ^{75}Se recovery was 101 ± 0.5% at 60°C and 98 ± 4.1% at 100°C. For liver, ^{75}Se began to volatilize at temperatures above 120°C (Figure 1). When the liver was dried as described in a microwave method (15), recovery of ^{75}Se was 107 ± 1.0%.

Acid Mixture for Digestion

The suitability of various HNO_3–$HClO_4$ mixtures in digesting 2 mL blood containing metabolically incorporated ^{75}Se was examined. The percentage recovery of ^{75}Se after heating to fumes of $HClO_4$ was 106 ± 1.5, 105 ± 2.9, and 104 ± 3.3 for HNO_3-$HClO_4$ ratios of 5:3 (7 mL), 5:2 (5

mL), and 4:1 (4 mL), respectively. (Figures in brackets indicate volume of acid used.) The 4:1 ratio was selected for all subsequent investigations. The mean volume of residual $HClO_4$ after heating the 4:1 acid mixture to fumes of $HClO_4$ was 0.61 mL (coefficient of variation (CV) = 5.4%, number of determinations (n) = 10).

Reduction of Se(VI) to Se(IV)

After heating the digests to fumes of $HClO_4$, 0.5 mL 11.3M HCl was added to reduce Se(VI) to Se(IV). When these tubes were heated at 100, 150, and 210°C for 30 min each, the percentage of ^{75}Se recovery in blood was 102 ± 0.1, 103 ± 1.1, and 102 ± 1.1, respectively. The absence of ^{75}Se activity in 9M KOH which was used to trap the acid distillates confirmed that no ^{75}Se was lost during the acid digestion procedure. Attempts to reduce Se(VI) to Se(IV) by adding 0.5 or 2.0 mL 11.3M HCl without heating and left standing overnight led to a decreased extraction of ^{75}Se into the cyclohexane, viz., 83 ± 7.1 and 38 ± 8.9%, respectively, of that obtained from tubes heated at 150°C.

Types and Volumes of Diluents

The suitability of 5, 10, and 15 mL volumes of glass-distilled water and of 0.1M HCl for diluting the digest to a suitable volume for chelation and extraction of ^{75}Se was evaluated. With 5 mL volumes of each diluent, ^{75}Se recovery was erratic and was about 80% of that obtained with 15 mL glass-distilled water. Since glass-distilled water and 0.1M HCl gave similar recovery for ^{75}Se, 15 mL glass-distilled water was selected as the diluent. However, in subsequent investigations, it was decided to use 0.0025M EDTA solution as the diluent for the modified method.

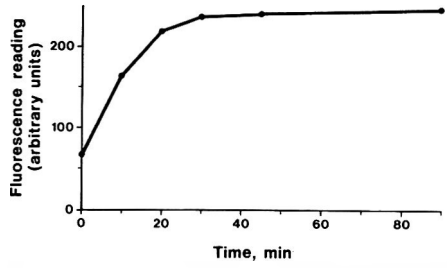

Figure 2. Effect of incubation time on fluorescence response. Conditions: temperature 60°C, pH 0.4, Se 20 μmol. Each point represents the mean of 4 determinations.

Effect of Incubation Time on Se-DAN Formation

An incubation temperature of 50°C required at least 50 min to be sure that formation of Se-DAN had gone to completion. As indicated in Figure 2, at 60°C, the fluorescence response reached a plateau at 30 min. To ensure greater tolerance margin, a 40-min incubation time was adopted for the modified method.

Effect of pH Adjustment

To determine the need for pH adjustment, the fluorescence response of Se standards obtained without pH adjustment was compared with those which had been adjusted to pH 1.8, using cresol red as the indicator. As illustrated in Tables 1 and 2, the tedious step of pH adjustment is not only unnecessary but also has the disadvantage of producing a high blank reading. After the

Table 1. Comparison of fluorescence response of Se standards obtained with and without adjusting pH of digests

Se standard, μmol/L	Fluorescence (arbitrary units)		
	pH not adjusted	pH adjusted[a]	pH not adjusted, simplified addition of reagents[b]
0.00	2	11	2
0.25	35	47	34
0.50	74	83	72
0.75	116	131	131
1.00	154	179	155
2.00	311	347	295
r^2	1.00	1.00	0.99
Y-intercept	−0.91	6.27	3.10
Slope	156.5	169.6	148.7

[a] pH adjusted to 1.8 using cresol red as indicator.
[b] Reagents added in following sequence: 2 mL EDTA–DAN (1 + 1), 5 mL cyclohexane. Cap, mix, and incubate.

Table 2. Comparison of Se results obtained with and without adjusting pH of digests

Sample	Se,[a] mean ± std dev. (n = 3) pH not adjusted	pH adjusted[b]	pH not adjusted, simplified addition of reagents[c]
Blood 1	0.41 ± 0.01	0.40 ± 0.02	0.38 ± 0.01
Blood 2	2.12 ± 0.02	2.09 ± 0.05	2.23 ± 0.10
Liver	14.15 ± 1.14	13.42 ± 0.33	14.9 ± 0.41

[a] μmol/L for blood; μmol/kg dry weight for liver.
[b] pH adjusted to 1.8 using cresol red as indicator.
[c] Reagents added in following sequence: 2 mL EDTA–DAN (1 + 1), 5 mL cyclohexane. Cap, mix, and incubate.

extraction, the mean pH of the aqueous phase was 0.4 (n = 10) for those tubes which had not been pH adjusted.

Addition of EDTA-DAN Combined Solution and Cyclohexane

In all the published methods for the fluorometric determination of Se, reagents for chelation and extraction of Se were generally added in the following sequence: EDTA, mix, pH adjustment, DAN, mix, incubate, cool, cyclohexane, mix to extract Se-DAN complex. To simplify the procedure, the feasibility of the following sequence was investigated: EDTA–DAN (1 + 1) solution, cyclohexane, cap, mix, incubate, and mix to extract Se-DAN complex. As shown in Tables 1 and 2 (last column), results were similar for both methods of reagent addition. However, EDTA–DAN solution is unstable and must be dispensed into culture tubes within 15 min of preparation, otherwise EDTA will crystallize in the solution.

Stability of Extracted Se-DAN Complex

After extraction of the ^{75}Se–DAN complex into cyclohexane, the organic phase was left in contact with the aqueous phase at room temperature either exposed to fluorescent light or away from fluorescent light (i.e., with tubes kept in a cupboard). The organic phase was counted for ^{75}Se activity at various time intervals. The results in Table 3 indicate that even after 16 h exposure to fluorescent light, there was negligible change in the ^{75}Se activity. Investigation on the effect of organic/aqueous phase contact time on fluorescence response, as distinct from ^{75}Se activity, revealed that while an overnight contact time did not affect the fluorescence response, a contact time of one week would result in an increase of a fixed level of fluorescence reading in all tubes including the blank. However, such apparent increase in fluorescence reading did not appear to affect the Se results.

Percentage of Se Extracted into Cyclohexane

When a second extraction was performed on the aqueous phase, negligible fluorescence reading was detected, suggesting that the first extraction is 100% efficient. However, with the ^{75}Se experiments it was found that only about 85% of the ^{75}Se was extracted.

Precision and Accuracy of the Modified Method

The within-batch variation of the modified method is about 2% while between-batch variation over a period of 2 years was less than 10% (Table 4).

Accuracy of the modified method was assessed by assaying certified Standard Reference Material and performing recovery tests. Tables 5 and 6 show that the modified method is accurate and applicable to determining Se in a wide range of biological materials.

Discussion

We found that at drying temperatures up to 120°C, no significant loss of ^{75}Se from liver was

Table 3. Effect of storage time on the activity of blood ^{75}Se-DAN extracted into cyclohexane and kept in contact with aqueous phase at room temperature in the dark

Time after extraction, h	^{75}Se activity (%), mean ± std dev. (n = 4)
0	100 ± 2.3
0.5	102 ± 1.5
1	101 ± 2.0
2	102 ± 2.3
16	106 ± 2.3
16[a]	107 ± 1.6

[a] Tubes exposed to fluorescent light.

Table 4. Precision of modified method over a 2-year period

Sample	No. of detns	Se[a]	CV, %
Blood 1	96	2.04	7.2
Blood 2	85	0.41	7.8
Liver 1	43	14.6	8.9
Liver 2	42	25.3	9.8

[a] μmol/L for blood; μmol/kg dry weight for liver.

Table 5. Determination of Se in quality control materials using the modified method

Material	Se, μmol/kg dry weight	
	Found[a]	Certified
IMVS Bovine Liver[b]	14.2 ± 0.3	13.9 ± 0.9
NBS Bovine Liver 1577	14.5 ± 0.3	13.9 ± 1.4
NBS Orchard Leaves 1571	0.9 ± 0.1	1.0 ± 0.1

[a] Mean ± std dev. (n = 3).
[b] Reference material used in an Australia–New Zealand interlaboratory survey (ref. 1).

Table 6. Percentage recovery of 1 μmol Se in various biological materials, using the modified method

Sample	Se (μmol/kg),[a] mean ± std dev. (n = 4)	% Recovery of 1 μmol Se
Barley	3.1 ± 0.15	101 ± 2.1
Wheat	3.6 ± 0.11	102 ± 3.8
Lucerne (alfalfa)	0.8 ± 0.05	103 ± 0.6
Poultry feed	2.2 ± 0.06	99 ± 3.5
Fish (*Arripis georgianus*)	36.3 ± 0.69	99 ± 1.1
Hair	7.9 ± 0.17	99 ± 0.8
Blood	2.0 ± 0.04	97 ± 2.0
Urine	0.4 ± 0.01	100 ± 2.2
Milk	0.2 ± 0.01	97 ± 0.9

[a] μmol/L for blood, urine, and milk.

observed. This is in contrast with other reports which showed that in oyster 30% of ^{75}Se was lost at 120°C (16). In blood and urine, no loss of ^{75}Se was observed at temperatures up to 200°C (17). Such variation in the drying temperature at which loss of Se occurred could be due to the presence of Se compounds with different volatilities in various biological materials. The apparent stepwise loss of Se shown in Figure 1 indicates that, at least in liver, Se may exist in several different forms.

Conflicting statements have been made on the suitability of HNO_3 and $HClO_4$ for digesting biological materials for Se determination. Michie et al. (12) stated that it was necessary to remove HNO_3 and $HClO_4$ from the digest to avoid interference with the formation of the Se-DAN complex. For the above reason, they advocated adding H_2SO_4 to the digest. In addition to H_2SO_4, some workers added compounds of molybdenum to the acid mixture for catalytic purposes (11, 18, 19). Criticism of the use of H_2SO_4 and molybdenum compounds include possible precipitation of DAN in the presence of sulfate, oxidation of DAN by molybdate leading to loss of sensitivity (8), and difficulty in purifying the sulfuric and molybdic acids (20). In addition, the "spitting" phenomenon when water is added to H_2SO_4 means that extra care has to be taken when diluent is added to the H_2SO_4 digest. A survey of the literature indicated that the majority of laboratories preferred to use HNO_3 and $HClO_4$ acid mixture for digestion of samples. Except for a few laboratories (9, 11, 21), the volume of the acid mixture recommended was too large for the Pyrex culture tube used in our modified method. Clinton (22) used 7 mL HNO_3–$HClO_4$ (5 + 2) acid mixture and claimed to complete the digestion in 1.5–2.5 h, but our experience indicated that at least twice that length of time was required to complete digestion. With our modified method, acid digestion was normally carried out unattended overnight. The 4 mL acid mixture used in this method is $\frac{1}{3}$ the volume recommended in the AOAC method (23) but is sufficient to digest up to 0.5 g of a wide variety of dried biological materials. In contrast with the claims that prolonged heating, e.g., for more than 10 min, of the acid digest to fumes of $HClO_4$ might lead to low recovery of Se (7), no significant loss of ^{75}Se was observed in this study. Stanton and McDonald (24) reported large selenium losses when HNO_3–$HClO_4$ digests from soils were accidentally allowed to evaporate to dryness. We encountered a similar accident with blood samples but did not observe any loss of ^{75}Se.

The requirements and conditions for the reduction of Se(VI) to Se(IV) are controversial. Basically, there are 3 groups of analysts, viz., those who did not perform reduction (9,11), those who performed reduction at room temperature (7, 22, 25), and those who performed

reduction with heating (6, 8, 10, 20, 21). Generally, HCl is the reducing agent used but some laboratories reported the use of H_2O_2 (12) or hydroxylamine (19). Our results indicated that attempts to reduce Se(VI) to Se(IV) with HCl without heating and left standing overnight would lead to poor precision and low recovery. Lalonde et al. (25) claimed to achieve good recovery of Se with an overnight reduction with HCl at room temperature. Detailed examination of these authors' methods showed that they might have already reduced the Se(VI) to Se(IV) in the earlier part of their digestion procedure when 0.2 mL 6M HCl was added to the acid mixture and heated 5 min at 150°C. Despite claims that Se could be lost during acid digestion, especially during the HCl reduction step, our results using 9M KOH to trap acid distillates indicated the contrary. Accordingly, the elaborate reflux adapters and closed system digestion apparatus used in many laboratories and the precautions of Lalonde et al. (25) that digestion must be carefully controlled to avoid changes of temperature by more than 10°C are unnecessary.

Because of the modified digestion method, it was necessary to re-assess the incubation conditions for the formation of Se-DAN complex. Most laboratories used an incubation temperature of 50°C for 20 to 35 min (6–8, 10, 11, 20). We preferred a 60°C incubation temperature because of the shorter incubation time required.

Our literature search revealed that all published fluorometric methods for the determination of Se involved the tedious step of pH adjustment for the formation of Se-DAN complex. However, with our modified method, we were able to eliminate this time-consuming and contamination-prone step of pH adjustment. It should be noted that the non-pH adjustment step recommended in our method might not be applicable to other fluorometric methods. The high blank reading obtained with the pH adjustment method when cresol red is used as an indicator could be due to the presence of residual fluorescent impurities in the indicator. The Se content of the cresol red was estimated to be 0.25 nmol Se/kg and this was considered an insignificant source of Se contamination. Optimum pH for the formation of Se-DAN complex has been reported to range from 1.1 (2) to 2.4 (12), with a pH range of 1.8–2.0 the most preferred (4–8, 10, 11, 20, 23, 26, 27). Michie et al. (12) reported that when the reaction mixture was heated 30 min at 60°C, the fluorescence response altered rapidly from pH 1.4 to 2.0 and the response became almost independent of acid from pH 2.0 to 3.5. On the other hand, Grant (21) showed that when the reaction mixture was heated 1 h at 40°C, the fluorescence response was independent of acidity in the pH range 1.1–2.5. The difference in optimum pH findings could be due to many factors, e.g., incubation temperature and time. In our modified method, the final pH of the reaction mixture post-extraction averaged 0.4 (CV 0.4%, $n = 10$). Although the modified method was not performed at the optimum pH of 1.5, at which the rate of Se-DAN formation was maximum (3), the complex formation was allowed to go to completion, as indicated in Figure 2. With the modified method, it should be noted that Se standards should undergo the complete analytical procedure as for samples so that operational conditions are closely matched for both groups.

In addition to the modifications mentioned, the addition of EDTA, DAN, and cyclohexane for chelation and extraction was simplified in the modified method. All the published fluorometric methods cited indicated the necessity of cooling the incubated mixture to room temperature before solvent addition, presumably to minimize evaporation of solvent on contact with hot aqueous phase. This problem was overcome by using culture tubes sealed with screw caps. Although this study showed that EDTA and DAN could be added as EDTA–DAN combined solution, it was noted that the combined solution is unstable, owing to the tendency of EDTA to crystallize in the strongly acidic environment. Subsequently, it was decided to separate EDTA from DAN and add EDTA to the reaction mixture as 0.0025M solution, replacing water as the diluent. With this modification we were able to eliminate the problem of EDTA crystallization and, at the same time, retain the simplicity of reagent addition.

Although many laboratories recommended that the chelation/extraction step be performed in diffuse or amber light (6, 10, 13, 20, 21, 23, 25–27), we have found this precaution unnecessary. In their kinetic study on the reaction of Se with DAN, Cukor and Lott (3) stated that when DAN was exposed to daylight or ultraviolet light, it decomposed slowly and after a period of several hours the brown precipitate of the DAN polymer began to form. It was calculated that under our operating conditions the length of time that the purified DAN would be exposed to laboratory light was about 20 min, a period too short to have significant contribution toward the background fluorescence reading, as confirmed in the blank readings.

Our findings on the stability of the Se-DAN complex in cyclohexane are in contrast with the report of Michie et al. (12), who found that extracted Se standards stored for 12 h showed a 10% decrease in fluorescence response. In our laboratory, organic and aqueous phases were often left in contact overnight before fluorescence readings were made and a significant increase in fluorescence from impurities was rarely observed. It was only with storage time at one week that fluorescent impurities from the degradation of DAN caused an increase in the background fluorescence reading. Nevertheless, because the level of fluorescent contaminants was similar for samples, Se standards, and blanks, the outcome of the Se determinations in the samples was unaffected.

The AOAC method (23) and others (7, 26) recommended washing the extract several times with 0.1M HCl to remove fluorescent impurities. Such a step was deemed unnecessary provided the DAN purification procedure was properly carried out.

Several analysts reported that traces of water in the solvent extract would affect the stability of Se-DAN complex or cause fluorescent interference. Methods used for removing traces of water from the solvent include centrifugation (9, 11, 23); centrifugation with anhydrous $CaCl_2$ added (20); filtration through silicone-treated paper (12), and storing the extract in a desiccator containing silica gel (21). Our results showed that water removal is an unnecessary step.

The lack of fluorescence response from the second extraction of the aqueous phase indicated the effectiveness of the extraction step in the modified method. However, in the ^{75}Se experiment, only about 85% of the total ^{75}Se activity could be accounted for in the organic phase. The incomplete recovery of ^{75}Se could not be explained by assuming incomplete digestion of organic matter and consequent failure to release all Se since conditions of digestion were considered sufficiently rigorous to destroy all organic matter, as was evident from the analyses of NBS SRMs. Grant (21) reported a similar observation and attributed the incomplete extraction of ^{75}Se to the reduction of selenite to elemental Se by DAN per se so that a proportion of the Se was not available for complex formation. The other possible explanation is based on the occurrence of self-radiolysis (28) where a portion of ^{75}Se-DAN could have decomposed by self-irradiation.

As shown in Tables 4, 5, and 6, the precision and accuracy of the modified method are satisfactory. The results in Table 6 indicate that the modified method is applicable to the determination of Se in a wide range of biological materials.

The culture tube used in our modified method has the advantages of reducing the quantity of laboratory glassware and chemicals used and the frequency of transferring sample solutions, and hence minimizes analytical errors. Criticism of the suitability of cyclohexane for solvent extraction because of its volatility was overcome by the screw-top feature of the culture tube. With the modified method, the screw top was put on immediately after cyclohexane addition and then was not taken off until the last step of the method when fluorometric readings were made. The screw top is superior to the glass stopper because it is not fragile, it is leak-proof, and does not get jammed in the socket of the tube.

In summary, with the aid of metabolically incorporated ^{75}Se, results obtained in this study have either disputed or overcome a number of criticisms and myths which have been laid against the fluorometric method for many years. The modified method, which can handle about 200 samples per batch, has been successfully used routinely in this laboratory for determining Se in several thousands of biological samples over the past several years.

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VITAMINS AND OTHER NUTRIENTS

Chloroform-Methanol Extraction Method for Determination of Fat in Foods: Collaborative Study

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A chloroform-methanol extraction method (complete extraction of fat in 3 min) for determining fat in processed and prepared foods has been studied collaboratively. Fourteen collaborators reported single replicate fat results on 7 samples representative of various food types and 2 spiked samples by the proposed method. Each sample was accompanied by a blind duplicate. For statistical purposes, the blind duplicates were treated as paired observations, and there were 2 laboratory outliers. There was a 97.9% agreement among the results from the remaining 12 collaborators and the Associate Referee for the unfortified samples. Recoveries of 93.8 and 98.3% were obtained on fortified samples, based on results obtained from 11 collaborators. The statistical analysis of the results indicate (ranges for standard deviations were S_r = 0.083-0.528, S_b = 0.101-0.379, S_d = 0.130-0.631, for fat values ranging from 1.58 to 26.91%) that this method is adequate for quantitating the fat content in a wide variety of processed foods for nutritional labeling. The method has been adopted official first action.

The fat content of food has received considerable attention in recent years from both nutritionists and medical scientists. This interest in fat can be attributed to its caloric contribution as well as its relationship to cholesterol which may be associated with certain forms of heart disease. This interest, allied with nutritional labeling, necessitates the need for more rapid, accurate, and precise methods for determining fat in foods.

Most AOAC methods in *Official Methods of Analysis* (1), although accurate and precise, are only applicable to specific food types. Because many of today's prepared foods cross boundaries of several food groups, it is often difficult for the analyst to decide which method is most appropriate.

In 1959, Bligh and Dyer (2) described a method for the complete extraction of the lipid fraction in a biological material by means of a chloroform–methanol extraction solvent system. Critical to the complete extraction of lipids, however, was the necessity to maintain the ratios of chloroform, methanol, and water in the initial and final steps of the extraction in the proportions of 1:2:0.8 and 2:2:1.8, respectively.

Lento and Daugherty (3) reported a modification of this method and showed that it was applicable to a wide variety of food types.

More recently, Lento and Daugherty (4) reported the results of a collaborative study which demonstrated the accuracy and applicability of this method to different food types.

The results of a second collaborative study, conducted to substantiate the applicability of this method for the determination of fat in prepared foods, are discussed in this report.

Collaborative Study

Seven food samples representing 4 food groups were selected and prepared for this study. One sample (vegetable soup puree) was prepared in a larger quantity than the rest. This sample was divided into 3 equal portions. Two of the portions were spiked with winterized vegetable oil at 2 levels (0.8 and 1.2 g oil/100 g). These 9 samples were divided into 2 equal portions, transferred separately to sealable pouches, and identified as follows: Samples 1–4, yogurt at 2 levels of fat; Samples 5–10, vegetable soup puree and 2 spiked samples; Samples 11 and 13, chicken frankfurters; Samples 12 and 14, beef frankfurters; Samples 15–18, cookie–bread crumb mixture at 2 levels of fat.

Fifteen collaborators were supplied with the 18 samples (identified only by the numbers 1–18). The collaborative kits also included a

The recommendation of the Associate Referee was approved by the General Referee and Committee D and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

This report of the Associate Referee, C. E. Daugherty, was presented at the 96th Annual International Meeting of the AOAC, Oct. 25-28, 1982, at Washington, DC.

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Table 1. Collaborative results for determination of fat (%) in foods [a]

	Sample A		Sample B		Sample C		Sample D		Sample E		Sample F		Sample G		Sample H		Sample I	
Coll.	1 [b]	3	2	4	5	6	7	8	9	10	11	13	12	14	15	16	17	18
1 [c]	2.16	2.16	1.51	1.60	1.52	1.51	1.81	1.92	2.45	2.39	23.71	23.56	25.66	25.34	9.64	9.77	14.22	14.29
2	2.77	2.73	2.16	2.18	1.55	1.59	2.36	2.43	2.61	2.66	25.49	25.40	27.20	26.04	9.79	10.48	15.17	15.92
3	2.85	2.82	2.13	2.20	1.29	1.18	2.22	2.47	2.99	3.04	24.99	24.32	27.25	27.80	10.08	9.97	14.45	14.64
4 [c]	3.72	3.52	3.11	2.60	1.58	1.96	2.86	3.02	3.44	3.44	27.42	27.50	27.14	27.34	10.16	10.73	15.06	15.20
5	2.72	2.72	2.10	2.08	1.54	1.57	2.15	2.16	2.49	2.54	24.86	25.10	26.50	26.14	9.62	9.64	14.45	14.43
6	2.89	2.85	2.90 [d]	2.65 [d]	2.06	1.90	2.50	2.40	3.11	2.87	26.27	25.40	25.87	25.87	9.92	9.73	14.49	14.29
7	2.60	2.62	1.82	2.03	1.41	1.62	2.35	2.34	2.64	2.67	24.44	24.50	27.00	27.68	9.72	10.26	14.56	15.38
8	2.67	2.72	1.99	1.99	1.44	1.52	2.17	2.25	2.64	2.80	23.50 [e]	25.51 [e]	26.32	26.34	10.52	9.93	15.45	14.90
9	2.86	2.90	2.30	2.16	1.63	1.64	2.21	2.29	2.68	2.59	25.50	26.20	26.60	27.70	10.20	10.30	15.30	15.50
10	2.50	2.47	1.85	1.93	1.32 [f]	1.38 [f]	1.92 [d]	1.88 [d]	2.17 [f]	2.28 [f]	24.56	25.41	26.94	27.83	9.96	9.62	14.49	14.43
11	3.10	2.54	1.76	2.18	1.86	1.25	2.49	2.39	2.86	2.64	26.47 [d]	24.66 [d]	27.66	26.47	9.85	10.10	14.83	14.80
12	2.50	2.39	1.84	1.75	1.90	1.85	2.47	2.58	2.99	2.95	24.54	25.14	26.91	27.05	9.52	9.34	14.94	14.87
13	2.76	2.81	2.04	2.07	1.46	1.42	2.35	2.27	2.76	2.91	25.35	25.43	27.40	26.78	9.48	9.76	14.74	14.55
14	2.78	2.62	2.10	2.01	1.49	1.57	2.30	2.12	2.61	2.75	25.32	24.95	27.60	26.81	9.76	9.34	14.42	14.50

[a] A = yogurt; B = yogurt; C = veg. soup puree; D = veg. soup puree fortified; E = veg. soup puree fortified; F = chicken frankfurter; G = beef frankfurter; H = cookie–bread crumb; I = cookie–bread crumb.

[b] Collaborative sample number.

[c] Rejected by Youden's ranking test (5).

[d] Outliers rejected by the Dixon test (5).

[e] Results rejected by the Cochran test (5)

[f] Results excluded to maintain complete block of data for the recovery experiment.

Table 2. Ranked collaborator results

Coll.	Sample A	B	C	D	E	F	G	H	I	Coll.[a] score
1	14	14	9.5	14	13	14	14	10	14	116.5
2	7	4	5	5	10.5	5	10	4	1	51.5
3	4	5	14	6.5	2	10	1	5	9	56.5
4	1	1	3	1	1	1	4	1	5	18
5	8	6	6.5	12	12	8	12	11	12	87.5
6	3	2	1	3	3	3	13	9	13	50
7	11	11	9.5	6.5	9	13	3	6	2	71
8	10	9	11	10.5	7	11	11	3	4	76.5
9	2	3	4	9	10.5	2	6	2	3	41.5
10	12	12	13	13	14	12	2	8	10.5	96.5
11	5	10	6.5	4	6	4	8	7	7	57.5
12	13	13	2	2	4	9	9	14	6	72
13	6	7.5	12	8	5	6	7	12	8	71.5
14	9	7.5	8	10.5	8	7	5	13	10.5	78.5

[a] Acceptable range for 14 collaborators and 9 samples (33–102).

practice sample, a 1 oz packet of enzyme (Clarase 40,000), a copy of the method, a list of instructions, and a data sheet. The collaborators were instructed to make several runs with the practice sample to familiarize themselves with the method. The fat content of this sample (4.54% ± 0.21) was also provided. The collaborators were then asked to analyze each of the 18 samples singly by the proposed method.

Fat in Foods

Chloroform-Methanol Extraction Method

Official First Action

(Applicable to processed and prepd foods. Not applicable to meat.)

(*Caution:* See **51.004, 51.011,** and **51.056.**)

43.D01 *Apparatus and Reagents*

(a) *Blending assembly.*—Semi-micro, stainless steel blending assembly for Waring blender (American Scientific Products Co., Cat. No. S8395-1, or equiv.).

(b) *Enzyme soln.*—1% Clarase 40,000 in 0.5M Na acetate soln. Suspend 10 g Clarase 40,000 (Miles Laboratories, Inc.) in 200 mL 0.5M Na acetate soln in 1 L vol. flask, dil. to vol. with 0.5M Na acetate soln, and mix thoroly. (Stable at least 7 days when stored at 4°.)

43.D02 *Determination*

Accurately weigh ca 5 g well minced sample (3 g if >10% fat) into 50 mL digestion tube, add enough enzyme soln so that total H_2O content (enzyme soln added plus original moisture in sample) is 32 mL. Shake gently until sample is thoroly mixed with enzyme soln and place tube in 45–50° H_2O bath 1 h. Mix thoroly and quant. transfer digest to blending assembly with 80.0 mL MeOH and 40.0 mL $CHCl_3$.

Cover and blend 2 min at high speed. Remove cover, add 40.0 mL $CHCl_3$, cover, and blend 30 s. Remove cover, add 40 mL H_2O, cover, and blend addnl 30 s.

Transfer ext to 250 mL polypropylene centrf. bottle and centrf. 10 min at ca 3000 rpm (when rotor diam. is 11.5 in.) to clarify bottom $CHCl_3$ layer. Pipet 20 mL $CHCl_3$ ext into tared 100 mL beaker. (Care should be taken to prevent transferring aq. phase.) Evap. to dryness on steam bath in fume hood. Dry fat residue in 101° oven to const wt (ca 30 min), cool in desiccator to const wt (ca 30 min), and weigh. Calc. % fat = (g residue/g sample) × 4 × 100.

(*Note:* Ratio of $CHCl_3$–MeOH–H_2O is critical for quant. extn of fat. This may necessitate detn of moisture content of sample to optimize amt of H_2O (as enzyme soln) in initial extn step. Reference source such as USDA Agriculture Handbook No. 8, *Composition of Foods*, or USDA Agriculture Handbook No. 456, *Nutritive Value of American Foods*, is adequate for this purpose. Also use as guide to pre-estimate fat content so that proper sample wt can be chosen for analysis.)

Results and Discussion

Fourteen collaborators reported single replicate results on the 18 samples by the proposed method (Table 1). For statistical purposes, the blind duplicates were treated as paired observations and the results of the 9 samples were subjected to Youden's ranking criteria (5) for outlying laboratories. According to Youden (5),

the acceptable range of scores for ranking 14 laboratories and 9 samples is 33–102. As shown in Table 2, Laboratory 1 was rejected with a score of 116.5, indicating consistently low results. Likewise, Laboratory 4 was rejected with a score of 18, indicating consistently high results. The remaining data were ranked and no additional extreme rank scores were observed.

The remaining 12 collaborator results were then subjected to Dixon's test (5) for individual outliers. Collaborator 6 reported high values for Sample B and Collaborator 10 reported low values for Sample D (see Table 1). The results reported by Collaborator 10 for sample E, although not rejected by the Dixon test, were lower than the remaining collaborators. Therefore, to maintain a complete block of the data for calculating the percent recovery of the fortified samples (Samples D and E), Collaborator 10's results for Samples C, D, and E were excluded from the statistics.

Cochran's test (5) was used to test for variation between duplicates. Collaborators 8 and 11 reported duplicate results for Sample F that were significantly different and outside the 95% probability of being sufficiently homogeneous (see Table 1).

Table 3 contains the statistical summary of the collaborative results. The statistical analyses were calculated separately for each of the 9 samples in Table 1, using the following procedure: S_r, or precision standard deviation, was calculated according to Youden (5) by the formula $S_r = \sqrt{\Sigma d^2/2N}$, where d^2 is the square of the difference between the independent analyses (blind duplicates) and N is the number of collaborators. S_b (bias), or between-laboratory variation was calculated according to Steiner (5), using the between-laboratory mean square (MS_L) from the analysis of variance and substituting in the following formula:

$$S_b = \sqrt{\frac{MS_L - S_r^2}{r}}$$

where r is the number of replicates ($r = 2$) and S_r is the random error as determined above. S_d, the overall or reproducibility standard deviation, was calculated by substituting in the following equation:

$$S_d = \sqrt{S_r^2 + S_b^2}$$

According to Youden (5), the random error (S_r) is usually $\frac{1}{2}$ to $\frac{1}{3}$ as large as the (S_b) between-laboratory variation. However, when blind duplicates are used, as in this study, there is less

Table 3. Statistical summary of collaborative results for determination of fat (%) in food by chloroform–methanol extraction

Statistic[a]	Sample A	B	C	D	E	F	G	H	I
n	24	22	22	22	22	20	24	24	24
Mean	2.72	2.03	1.58	2.33	2.76	25.16	26.91	9.87	14.81
S_r	0.123	0.111	0.147	0.083	0.093	0.387	0.528	0.261	0.267
S_b	0.112	0.107	0.173	0.101	0.153	0.379	0.345	0.200	0.347
S_d	0.166	0.153	0.227	0.130	0.179	0.541	0.631	0.329	0.437
RSD_r,%	4.53	5.45	9.29	3.56	3.38	1.54	1.96	2.65	1.80
RSD_b,%	4.11	5.22	10.95	4.31	5.54	1.50	1.28	2.82	2.34
RSD_d,%	6.12	7.55	14.36	5.59	6.49	2.15	2.34	3.33	2.95

[a] n = number of results representing each sample.
S_r = random error (precision standard deviation).
S_b = systematic error (between-laboratory variability).
S_d = combined error (reproducibility standard deviation).
RSD_r = relative standard deviation (repeatability coeff. of var.).
RSD_b = relative standard deviation (between-laboratory coeff. of var.).
RSD_d = relative standard deviation (reproducibility coeff. of var.).

Table 4. Recovery of vegetable oil (g/100 g) added to vegetable soup puree

Sample	Amt contributed by Sample C	Amt added	Total expected	Total found	Total amt recd	Recovery of added amt, %
D	1.58	0.80	2.38	2.33	0.75	93.8
E	1.58	1.20	2.78	2.76	1.18	98.3

Table 5. A comparison of results (% fat) for the determination of fat by the proposed method and official final action methods

Sample	Proposed[a] method	S_d	RSD, %	Official[a] methods	S_d	RSD, %
A (yogurt)	2.77	0.099	3.57	2.89[b]	0.061	2.11
B (yogurt)	2.18	0.073	3.35	2.08[b]	0.093	4.47
C vegetable soup puree	1.63	0.136	8.34	1.60[c]	0.050	3.12
D fortified vegetable puree	2.42	0.080	3.30	2.47[c]	0.097	3.93
E fortified vegetable puree	2.97	0.030	1.01	2.92[c]	0.076	2.60
F chicken frankfurters	25.12	0.363	1.45	24.69[d]	0.187	0.76
G beef frankfurters	27.18	0.384	1.41	26.96[d]	0.336	1.25
H cookie–bread crumb mixture	10.03	0.258	2.57	10.00[c]	0.182	1.82
I cookie–bread crumb mixture	14.90	0.188	1.26	14.81[c]	0.270	1.82

[a] All results are represented by 4 replicate determinations.
[b] Official method **16.256** (1).
[c] Official method **14.019** (1).
[d] Official method **24.005**[a] (1).

Table 6. Comparison of collaborative results with Associate Referee results (% fat) for determination of fat by proposed method

Sample	Collaborators (mean value)	Associate Referee (mean value)	% Agreement
A	2.72	2.77	98.2
B	2.03	2.18	93.1
C	1.58	1.63	96.9
F	25.16	25.12	100.2
G	26.91	27.18	99.0
H	9.87	10.03	98.4
I	14.81	14.90	99.4
			Av. 97.9

bias in the experiment and the dimension of the random error approaches that of the systematic error (see Table 3). Nevertheless, with the exception of Sample C, these statistical parameters are consistent with what would be ordinarily expected for foods containing these levels of fat. On the other hand, the relatively high variation in results for Sample C was not determined.

However, this sample (vegetable soup puree) which was spiked at 2 levels with winterized vegetable oil (0.8 and 1.2 g oil/100 g) yielded mean recoveries of 93.8 and 98.3% (see Table 4), respectively, as reported by 11 collaborators.

Also, the fat results obtained on all 9 samples by the Associate Referee, comparing the proposed method with official final action methods, are in good agreement (see Table 5). Furthermore, there was a 97.9% agreement between the mean results from the collaborators (Table 3) and the mean results obtained by the Associate Referee (Table 5) for the proposed method on the 7 unfortified samples (see Table 6).

There were no adverse comments received from the collaborators. However, several collaborators suggested that more specific information be included in the procedure regarding centrifuge speed and the time required to cool the oven-dried fat residue. These comments were regarded as important enough to make the necessary amendments to the method.

Conclusions and Recommendation

This study has demonstrated that the precision, reproducibility, and accuracy of the pro-

posed method are adequate for quantitating the fat content in processed and prepared foods for nutritional labeling. Therefore, the Associate Referee recommends that this chloroform-methanol extraction method for the determination of fat in food be adopted official first action.

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Effect of Heat Treatment on Dietary Fiber: Interlaboratory Study

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The effects of thermal treatments on the dietary fiber composition of cereal and potato samples were studied at 8 laboratories using different analytical methods. Thermal treatments included extrusion cooking for cereals, and boiling and frying for potatoes. No changes in the amounts of dietary fiber or starch were observed in the extruded samples. Heat-treated potato samples contained significantly more water-insoluble dietary fiber (cellulose) and less starch than did raw potato. However, this may be due, at least in part, to the sample preparation procedure rather than the heat treatment alone. The study indicates that gravimetric and sequential hydrolysis methods give similar results, but the variation in most cases is still wide. Further standardization of methods is evidently needed, especially if legislative measures for setting limits on the fiber content of foods are to be introduced. Standardization of starch analysis should also be of primary importance in future work on carbohydrate methodology. This can be concluded from the variation in results concerning this extremely important food constituent. It should be noted that the sample matrix in the present study was simpler than that of complex meals or diets.

The European Economic Community (EEC) Committee on Medical Research sponsored a meeting on dietary fiber analysis in Cambridge, UK, in December 1978 (1). The discussions focused on several analytical problems, one of which was the effect of technological procedures on the carbohydrate composition in foods. It was agreed that a collaborative study should be arranged to compare different analytical methods, especially in the analysis of some processed foods, and to study the effect of some technological operations on the dietary fiber (DF) content of foods.

Interlaboratory Study

Eight laboratories active in dietary fiber analysis were invited to participate in the study. The operations studied were 3 different thermal treatments of potato, and extrusion cooking of wheat whole grain meal and wheat flour under 2 different sets of conditions. The study was organized by the authors' laboratory.

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Sample Preparation

Sample processing and pretreatment conditions are given in Table 1. No pretreatment was required for the flour samples. The extruded materials were further ground in a hammer mill (particle size <0.5 mm). The raw and processed potato samples were freeze-dried before being homogenized in a blender. Samples (50 g) were vacuum-packed into polyethylene bags and mailed to the participants.

Methods

Individual analytical methods used by the 8 laboratories are presented briefly. In all laboratories, samples were analyzed as received without additional sample preparation. Dry matter, ash, and nitrogen determinations, if done, followed general standard procedures.

Laboratory A.—Dietary fiber was measured gravimetrically after enzymatic digestion (2, 3), omitting, however, the extractions with chloroform–methanol and with 80% ethanol. Pressure cooking before digestion was carried out at 120°C for 30 min. Enzyme solutions of pancreatin–glucoamylase were filtered. Starch was measured as glucose in the digestion filtrate.

Laboratory B.—Dietary fiber analyses were performed by using a rapid enzymatic, gravimetric method (4). The method requires 15 min incubation at 95°C with a thermostable amylase (Termamyl), a 1 h pepsin step at pH 1.5, and a 1 h pancreatin step at pH 6.8. Insoluble dietary fiber is then separated by filtration in G2 crucibles with Celite 545 filter aid. Soluble components are precipitated from the filtrate with 4 volumes of 95% ethanol and recovered by a second filtration.

Total dietary fiber was also analyzed by using a method (B_A) specifying the 15 min Termamyl

Table 1. Processing conditions for cereal and potato samples[a]

Conditions	Wheat flour (ca 0.5% ash)			Wheat whole meal		
	Untreated	Extruded 1	Extruded 2	Untreated	Extruded 1	Extruded 2
Screw velocity, rpm	—	150	200	—	150	200
Flour input, g/min	—	350	200	—	350	200
Moisture, %	—	15	15	—	15	15
Mass temperature, °C	—	161	168	—	163	180

	Potato			
	None (raw)	Boiled	Pressure cooked	French fried
Temperature, °C	—	100	121	180
Time, min	—	30	25	4

[a] Cereals processed by extrusion cooking using twin screw extruder Creusot Loire BC 45 (Technical Research Centre of Finland, Food Research Laboratory). Extruded materials ground in hammer mill.

Potatoes (var. Bintje) were washed and peeled, french fries were also cut into pieces. (Cooking water was discarded.) Raw and processed samples were freeze-dried and crushed, french fries were also extracted with petroleum ether. Samples were vacuum-packed into laminated polyethylene bags.

step, a 60 min protease incubation with protease P-5380, and, finally, 30 min incubation with a glucoamylase (Agidex). After the enzyme incubations, the sample is immediately precipitated with 4 volumes of 95% ethanol, and both originally insoluble and precipitated soluble components are recovered in a single filtration.

Starch was assayed by using Boehringer's amyloglucosidase and assay of free glucose. The samples were not extracted before analysis.

Laboratory C.—In the method used, a modification of Theander and Åman (5), the sample is extracted with 80% ethanol and chloroform. The residue is treated with a thermostable bacterial amylase in acetate buffer (pH 5.0, 0.5 h, 96°C) and then incubated with amyloglucosidase (16 h, 60°C). The water-insoluble fraction, isolated by centrifugation, is hydrolyzed with 72% H_2SO_4, and the water-soluble fraction, obtained by dialysis and freeze-drying, is hydrolyzed with 0.5M trifluoroacetic acid (16 h, 96°C). The neutral sugar composition is determined as alditol acetates by gas-liquid chromatography (GLC), and the resulting Klason lignin is determined gravimetrically. Both fractions were also analyzed for uronic acid content by a rapid decarboxylation method.

For starch determination, the sample is extracted by using ethanol–chloroform and treated with Termamyl (pH 5.0, 0.5 h, 96°C) and amyloglucosidase (16 h, 60°C) followed by glucose analysis using glucose-oxidase.

Laboratory D.—A modified Southgate-Englyst method was used (6). The sample is incubated with amyloglucosidase in an acetate buffer (pH 5, 16 h, 48°C) and centrifuged. The residue is sequentially hydrolyzed with 1M H_2SO_4 and 72% H_2SO_4, and analyzed by GLC as aldonitrilo acetates for water-insoluble, noncellulosic polysaccharides and cellulose, and gravimetrically for lignin. The supernate is analyzed for starch and water-soluble polysaccharides, the former as glucose, and the latter after acid hydrolysis as neutral sugars (GLC) and uronic acid (colorimetric determination).

Laboratory E.—In the Southgate method (7), free sugars are extracted with 85% alcohol. The residue is hydrolyzed with amyloglucosidase for starch determination. Water-soluble polysaccharides are precipitated with alcohol from the hydrolysate. The water-insoluble residue is subjected to dilute acid hydrolysis (water-insoluble, non-cellulosic polysaccharides) and to concentrated acid hydrolysis (cellulose). The liberated sugars are determined colorimetrically. Insoluble residue represents lignin (gravimetric determination).

Laboratory F.—Total insoluble dietary fiber was determined according to the procedure of Thomas (8, 9). The neutral detergent method was also employed (10). The gravimetric method of Thomas includes 2 enzymatic hydrolyses (pancreatin, pH 7, 5 h, 45–50°C, and rhozyme for amylolysis (pH 4.9, 18 h, 50°C), filtration, drying, and ashing.

Laboratory G.—Analyses were performed on the Tecator Fibertec (Höganäs, Sweden) using a sequential extraction with neutral detergent, acid detergent, and 72% H_2SO_4 (11). This procedure gives the amounts of lignin, cellulose, and hemicellulose, which together represent the total cell wall matrix less pectins (12).

Table 2. Effect of heat treatment on total dietary fiber (% of dry weight)

Sample	Laboratory							$\bar{x}$	s	CV, %
	A	B	B_A[a]	C	D	E	H			
Wheat flour										
Untreated	2.9	2.8	3.8	3.6	3.6	4.0	7.1	4.0	1.6	40
Extruded 1	5.3	3.6	3.6	4.0	3.9	2.5	6.3	4.3	1.3	31
Extruded 2	3.3	3.8	3.7	4.9	3.6	4.3	6.7	4.4	1.3	28
Wheat whole meal										
Untreated	9.3	12.2	12.7	9.3	10.4	11.6	17.0	11.6	2.9	25
Extruded 1	11.1	12.3	12.2	10.1	10.7	11.7	16.9	12.1	2.5	20
Extruded 2	9.1	11.3	11.7	11.0	10.9	12.7	14.9	11.7	1.9	17
Potato										
Raw	5.8	5.9	5.8	7.0	4.8	6.4		6.0[b]	0.8	14
Boiled	6.4	8.3	7.5	7.7	7.9	10.0		8.1	1.3	16
Pressure cooked	6.8	7.5	7.8	8.3	7.2	10.0		8.0	1.3	16
French fried		7.0	6.7	7.4	6.7	8.6		7.4	0.8	11

[a] Lab. B reported results for 2 methods. Results for B_A not included in calculations to avoid doubling the weight of results from Lab. B.

[b] The DF content of raw potato is significantly lower than that of boiled ($P < 0.02$), pressure cooked ($P < 0.01$), or french fried potatoes ($P < 0.05$).

Laboratory H.—The method (13, 14) consists of extraction (hot 90% ethanol), extensive ball-milling (15 h, 2°C), gelatinization (pH 7, 3 h, 85–90°C), and enzymatic treatments with α-amylase and pullulanase (37°C, 17 h). The residue is hydrolyzed (1M H_2SO_4 and 72% H_2SO_4), and the liberated neutral sugars are determined by GLC as alditol acetates, and as uronic acids after reaction with *m*-phenyl phenol. Starch is obtained as the weight difference between the residues before and after enzymatic hydrolysis.

Results and Discussion

In this collaborative study, every laboratory followed an individual analytical method. Laboratories A, B, and F used gravimetric methods; C, D, E, and H used sequential hydrolytic, and F and G used detergent methods. This explains the large number of fractions reported. However, no single fraction was reported by all laboratories. This complicates the interpretation, although it may still allow some comparison among methods. The results of fiber constituents and starch are given on the polymeric basis.

Total Dietary Fiber.—Especially in flour samples, the coefficients of variation are rather high, usually between 20 and 40% (Table 2). However, these are heavily influenced by the results of Laboratory H. Raw potato contained significantly less ($P < 0.05$; *t*-test) total DF than did

Table 3. Effect of heat treatment on water-insoluble dietary fiber (% of dry weight)

Sample	Laboratory					$\bar{x}$	s	CV, %
	A	B	C	D	F			
Wheat flour								
Untreated	2.4	1.9	2.7	2.6	1.3	2.2	0.6	26
Extruded 1	4.6	1.7	3.4	2.4	0.4	2.5	1.6	64
Extruded 2	2.7	0.8	3.0	2.1	0.3	1.8	1.2	66
Wheat whole meal								
Untreated	8.1	10.5	8.4	9.2	10.4	9.3	1.1	12
Extruded 1	10.0	10.1	9.5	9.2	9.8	9.7	0.4	4
Extruded 2	8.1	7.8	9.8	8.6	7.2	8.3	1.0	12
Potato								
Raw	2.6	3.5	5.8	3.3	2.2	3.5[a]	1.4	40
Boiled	3.3	5.8	6.5	6.2	4.1	5.2	1.4	27
Pressure cooked	3.6	5.4	7.1	5.6	4.4	5.2	1.3	25
French fried		5.4	6.0	5.3	3.5	5.1	1.1	21

[a] The water-insoluble DF content of raw potato is significantly lower than that of boiled ($P < 0.02$), pressure cooked ($P < 0.01$), or french fried potatoes ($P < 0.05$).

Table 4. Effect of heat treatment on neutral detergent fiber (% of dry weight)

Sample	Laboratory F	Laboratory G
Wheat flour		
Untreated	0.7	1.1
Extruded 1	0.3	0.4
Extruded 2	0.4	0.9
Wheat whole meal		
Untreated	8.5	11.3
Extruded 1	8.3	9.4
Extruded 2	7.8	7.7
Potato		
Raw	1.9	1.7
Boiled	9.5	3.4
Pressure cooked	3.2	3.2
French fried	2.2	5.4

processed potatoes. This may be caused by the sample preparation procedure (see starch). Extrusion cooking did not significantly affect total DF contents.

Water-insoluble Dietary Fiber.—Standard deviations and coefficients of variation in Table 3 show wide analytical variation, especially in the low DF content samples. However, these statistics may be of questionable value, because the solubility of DF seems to depend on the method used. (The same is true of results for water-insoluble DF.) The heat-treated potato samples contained more insoluble DF than did raw potato ($P < 0.05$). No significant differences were evident between the untreated and extruded cereal samples.

Neutral Detergent Fiber (NDF).—In comparison with water-insoluble DF (Table 3), this analysis gave low values for wheat flour and potato samples (Table 4). However, the levels of NDF and water-insoluble DF for wheat whole meal were similar. The NDF values for extruded cereals tended to be lower than those for untreated samples.

Water-soluble Dietary Fiber.—Extrusion cooking seemed to slightly increase the soluble fraction in the cereal samples, although the differences were not statistically significant (Table 5). The quantity of soluble DF in potato remained constant.

Cellulose.—Cellulose content of processed potatoes was significantly greater ($P < 0.01$) than that of raw potato (Table 6). No differences were evident between untreated and extruded cereal samples. Processing affected neither the noncellulosic polysaccharide nor the lignin content of the samples (Table 7).

Starch—Starch is the main constituent of all samples in the present study. Its complete enzymatic digestion is of crucial importance in DF analysis. The results (Table 8) indicate that there was some disagreement among laboratories with regard to starch levels of the samples. For instance, untreated wheat flour was found to contain 69–79% starch. The means of the untreated flour samples were 75.6% for wheat flour and 63.5% for whole meal. These values may be slightly low, because the sum of all major constituents (ash, protein, and fat estimated according to the food composition tables) is 2–5% below 100%. This suggests that the analytical methods for starch may not be sufficiently standardized at present.

The starch content of processed potatoes was significantly lower than that of raw potato. This suggests that some of the starch in the potato samples had become undigestible by amylolytic enzymes, and this had caused the observed in-

Table 5. Effect of heat treatment on water-soluble dietary fiber (% of dry weight)[a]

Sample	Laboratory A	Laboratory B	Laboratory C	Laboratory D	$\bar{x}$	s	CV, %
Wheat flour							
Untreated	0.5	1.0	0.9	1.0	0.9	0.2	27
Extruded 1	0.6	1.9	0.6	1.5	1.2	0.7	55
Extruded 2	0.6	3.0	1.9	1.6	1.8	1.0	55
Wheat whole meal							
Untreated	1.2	1.7	0.9	1.2	1.3	0.3	25
Extruded 1	1.2	2.1	0.6	1.4	1.3	0.6	48
Extruded 2	1.0	3.6	1.2	2.3	2.0	1.2	60
Potato							
Raw	3.2	2.4	1.2	1.5	2.1	0.9	43
Boiled	3.1	2.4	1.2	1.7	2.1	0.8	39
Pressure cooked	3.2	2.1	1.2	1.6	2.0	0.9	43
French fried		1.6	1.4	1.4	1.4	0.1	8

[a] No significant differences within groups.

Table 6. Effect of heat treatment on cellulose (% of dry weight)

Sample	Laboratory				$\bar{x}$	s
	C	D	E	G		
Wheat flour						
Untreated	1.1	0.5	Tr[a]	0.3		
Extruded 1	1.1	0.2	Tr	0.1		
Extruded 2	1.2	0.2	0.3	0.2		
Wheat whole meal						
Untreated	2.2	1.8	1.6	2.1	1.9	0.3
Extruded 1	2.1	1.6	1.9	1.9	1.9	0.2
Extruded 2	2.3	1.6	1.8	1.8	1.9	0.3
Potato						
Raw	2.4	1.4	2.1	1.5	1.9[b]	0.5
Boiled	4.1	3.3	3.9	2.6	3.5	0.7
Pressure cooked	4.4	3.1	3.9	2.6	3.5	0.8
French fried	3.4	2.4	3.3	2.5	2.9	0.5

[a] Tr = Trace.
[b] The cellulose content of raw potato is significantly lower ($P < 0.01$) than that of processed potatoes.

crement in the water-insoluble DF (cellulose) fraction. However, the change may not be caused by heat treatment alone, but rather by heat treatment combined with subsequent sample preparation steps (freeze-drying, storage). Heat-treated starch may be prone to retrogradation during drying, and the product is less readily hydrolyzed than freshly gelatinized starch. Since the analytical procedure for raw potato also includes a heat treatment, the starch content of raw and boiled potatoes would not be expected to be very different. On the other hand, some of the water-soluble dry matter was undoubtedly lost into cooking water. This could also explain, at least in part, the observed increment in the total DF of processed potato samples, perhaps also some of the decrease of starch values.

The present study shows that extrusion cooking caused no significant changes in carbohydrate composition of different wheat flours. Extrusion conditions 1 (Table 1) are considered normal whereas conditions 2 are moderately excessive. Neither caused clearcut changes in total DF. Extrusion cooking may slightly increase the share of water-soluble fiber. This is suggested by results on both water-soluble fiber (Table 5) and detergent fiber (Table 4), although the differences are not statistically significant. In processed potatoes, levels of total DF, water-insoluble DF, and cellulose were higher, and levels of starch are lower than in raw potato. However, this may reflect to some extent the effect of the sample preparation procedure as a whole, rather than just the effect of heat treatment.

Table 7. Effect of heat treatment on noncellulosic polysaccharides (NCP) and lignin (% of dry weight)

Sample	NCP				Lignin				
	Laboratory				Laboratory				
	C	D	E	G[a]	C	D	E	G	H
Wheat flour									
Untreated	2.4	2.9	4.4	9.5	0.1	0.2	0	0.2	0.7
Extruded 1	2.2	3.3	2.8	0.6	0.7	0.4	0	0.1	0.8
Extruded 2	3.2	3.1	4.4	0.6	0.5	0.4	0	0.1	1.3
Wheat whole meal									
Untreated	5.7	7.6	10.7	8.2	1.4	1.1	0.5	1.0	3.5
Extruded 1	5.3	6.6	10.5	6.5	2.7	1.1	0.5	1.0	3.8
Extruded 2	5.9	7.7	11.3	4.9	2.8	1.6	1.0	1.0	3.6
Potato									
Raw	3.5	3.0	5.3	0.2	1.1	0.3	0	0	
Boiled	2.8	4.2	7.6	0.8	0.8	0.4	0	0	
Pressure cooked	3.1	3.8	7.5	0.6	0.8	0.3	0	0	
French fried	3.2	5.5	6.5	2.2	0.8	0.5	0	0.7	

[a] Hemicellulose.

Table 8. Effect of heat treatment on starch (% of dry weight)

Sample	Laboratory							$\bar{x}$	s	CV, %
	A	B	C	D	E	F	H			
Wheat flour										
Untreated	74.3	72.3	69.3	78.4	77.1	78.5	79.3	75.6	3.8	5.0
Extruded 1	76.1	75.6	73.6	74.9	76.7	78.5	75.2	75.8	1.5	2.0
Extruded 2	77.3	74.7	71.2	75.3	77.2	78.3	76.1	75.7	2.4	3.1
Wheat whole meal										
Untreated	62.1	59.5	57.0	68.0	63.2	64.4	70.0	63.5	4.5	7.1
Extruded 1	63.1	64.6	60.2	64.3	65.8	63.3	67.2	64.1	2.2	3.5
Extruded 2	63.7	61.0	61.4	67.7	65.5	63.6	70.1	64.7	3.3	5.1
Potato										
Raw	76.0	76.8	73.0	73.9	73.7	72.2		74.3[a]	1.8	2.4
Boiled	75.5	78.1	71.6	70.7	71.8	69.0		72.8	3.4	4.6
Pressure cooked	72.9	73.6	71.0	70.2	69.8	71.2		71.5	1.5	2.1
French fried		73.9	69.7	63.0	68.2	66.8		68.3	4.0	5.8

[a] The starch content of raw potato is significantly higher than that of boiled ($P < 0.1$), pressure cooked ($P < 0.01$), or french fried potatoes ($P < 0.02$).

Only a very limited comparison of methods can be made from the present results. The DF results obtained by using gravimetric methods appeared to be approximately equal to those obtained by using sequential hydrolysis methods. However, there was a high variation within the results of both groups. The NDF method tended to give low fiber values for samples with a very low fiber content and a very high starch content. The results of Laboratory H were consistently higher than those of other laboratories (Tables 2, 7, and 8), and they thus differed from the general line.

The conformity in fiber analyses may not be expected to improve unless methodology is standardized. The lack of a standard method is a serious analytical shortcoming to which a satisfactory solution is needed in the near future. Analysis of starch also seems to require further standardization.

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Nonaqueous Reverse Phase Liquid Chromatographic Determination of Vitamin D_2 in Multivitamin Tablets, Using Vitamin D_3 as Internal Standard

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The technique of nonaqueous reverse phase chromatography has been successfully applied to the separation of lipophilic vitamins D_2 and D_3. Separation is achieved with a Zorbax-ODS column and a mobile phase of 10% methanol in acetonitrile. A predetermined amount of vitamin D_3 internal standard is added to a portion of a powdered tablet sample, which is then extracted and partitioned with a mixture of dimethyl sulfoxide, water, methanol, and hexane. A portion of the hexane extract is concentrated, and purified by passing it through a disposable silica cartridge to remove interfering materials. The method should also be applicable to formulations containing vitamin D_3 where vitamin D_2 can then be used as the internal standard. A system suitability test is included in the method.

Vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol) have about the same anti-rachitic activity in humans, rats, and most mammalian species, and are the forms most commonly used in pharmaceutical preparations. They are usually prepared as starch-coated beadlets, gelatin-protected beadlets, or oily solutions.

The assay of vitamin D_2 or D_3 in multivitamin dosage forms presents several difficulties. Vitamin D is sensitive to heat, light, and oxygen, and when in solution, tends to isomerize to its precursor, pre-vitamin D. The extent of the isomerization is dependent on the conditions. Other fat-soluble vitamins such as vitamins A, E, and K may interfere in the assay since vitamin D is present in relatively small quantities. Minerals, various excipients, and antioxidants may further complicate the sample preparation steps.

Several techniques for assaying vitamin D have been reported in the literature. Among them is a biological assay using rachitic rats. However, the assay is expensive, time-consuming, and imprecise. Other methods including separation and identification of D vitamins from sterols and retinol by argentation thin layer chromatography (1), infrared spectrophotometry (2, 3), and gas-liquid chromatography (4) have been investigated with varying degrees of success. In the case of gas-liquid chromatography, D vitamins undergo thermal cyclization on-column yielding the isomeric pyro- and isopyrocalciferols. Quantitation is based on the area of the major peak or both peaks.

High performance liquid chromatography (HPLC) offers the most promise as a technique that would not have the problems noted above. HPLC allows separation of the D vitamins from the pre-vitamins, tachysterol, and other isomers (5–8). Several recent applications of HPLC for the determination of vitamin D from gelatin-protected beadlets (9), from resins, oil, and dry concentrates (10–12), from multivitamin preparations (12–14), from fish products (6), and from feeds and premixes (15) have been published.

The current compendial assay procedures (16) are also expensive, time-consuming, and imprecise. Our experience with normal phase HPLC and external standard quantitation for vitamin D assay indicated that significant variation in assay results was quite common. This is mainly because of the volume changes in standard and sample solutions as a result of evaporation from a small volume (e.g., 4 mL) of volatile solution (e.g., hexane), and the inconsistency of the presumably 100% recovery from the sample cleanup operation using re-usable columns, which may also introduce cross-contamination between samples.

One solution to these problems is the use of a disposable cleanup column to eliminate cross-contamination, and an internal standard to offset small nonreproducible losses during sample preparation. Since the molecular structures of vitamins D_2 and D_3, and hence their physical-chemical properties, are similar, vitamin D_3 is the ideal internal standard for vitamin D_2 assays and vice versa. The next challenge is the development of a chromatographic system capable of resolving vitamins D_2 and D_3 from all other impurities, excipients, and degradation products present in the sample.

A literature review suggests that normal phase HPLC does not separate vitamins D_2 from D_3.

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Instead, reverse phase HPLC using Zorbax-ODS (17, 18), Vydac-ODS (14), and Lichrosorb RP-18 (6) columns with methanol–water (>90% methanol) were reported to be the methods of choice for resolving vitamins D_2 and D_3. Either partial resolution or skewed peaks or very long assay time was reported, however, and none was suitable for direct application to our vitamin D_2 assays.

The new technique of nonaqueous reverse phase HPLC (19) was attempted and successfully applied to the resolution of vitamins D_2 and D_3. This paper describes a new approach to improve the precision and accuracy of vitamin D_2 assays via nonaqueous reverse phase HPLC with vitamin D_3 as internal standard.

METHOD

Reagents and Materials

(a) *Vitamin D_2 and vitamin D_3 standards.*—USP Reference Standards (30 mg/ampule, U.S. Pharmacopeial Convention, Inc., Rockville, MD 20852) as reference and internal standards, respectively. Alternatively, house working standards of vitamins D_2 and D_3, assayed against the respective USP Reference Standards, may be used.

(b) *Chemicals.*—Hexane, methanol, acetonitrile, ethyl acetate (Burdick and Jackson Laboratories, Inc.) and dimethyl sulfoxide (reagent grade).

(c) *Cleanup cartridges.*—Disposable silica cartridges (Waters Associates SEP-PAK, and Analytichem Bond-Elut).

(d) *Disposable filters.*—Acrodisc-CR, 0.45 μm (Gelman Sciences).

(e) *Syringes.*—Disposable, 5 or 10 mL (Becton, Dickinson & Co.).

(f) *Mobile phase.*—10% (v/v) methanol in acetonitrile. Percentage of methanol may be adjusted slightly to achieve satisfactory system suitability.

(g) *Internal standard stock solution.*—Accurately weigh ca 27 mg vitamin D_3 and dissolve with hexane in 100 mL low-actinic volumetric flask. Store unused stock solution under refrigeration for up to one week. Let solution reach room temperature before use.

(h) *Reference standard stock solution.*—Prepare vitamin D_2 stock solution in the manner as for internal standard stock solution.

(i) *Vitamins D_2 and D_3 working standard mixtures.*—Label three 250 mL low-actinic volumetric flasks A, B, and C. Pipet appropriate volumes of vitamins D_2 and D_3 into each flask (Table 1) such that concentration ratios are ca 0.3, 1.0, and 1.5, and concentration of D_3 at all levels is ca 400 IU/mL. Mix solutions thoroughly and store under refrigeration until use. When standards are needed, evaporate aliquots of hexane solutions to dryness under stream of nitrogen at room temperature. Dissolve and dilute residues to volume with mobile phase or acetonitrile.

Table 1. Three-level standard mixtures

Standard	Vol. stock solns, mL[a] D_2	Vol. stock solns, mL[a] D_3	Approx. concn levels, IU/mL D_2	Approx. concn levels, IU/mL D_3	Approx. concn ratios, R_c (D_2/D_3)
A	3	9	130	400	0.3
B	9	9	400	400	1.0
C	15	9	600	400	1.5

[a] Assuming that about 27 mg vitamin D_2 or D_3 is used per 100 mL stock solution prepared, and that vitamins D_2 and D_3 have 40 IU per microgram. If actual weight of vitamin D_2 or D_3 used deviates significantly (i.e., more than 10%), calculate appropriate volumes of stock solutions that should be used.

Apparatus

Liquid chromatograph.—Varian LC-5000 equipped with variable wavelength UV detector set at 265 nm (Perkin-Elmer LC-75), auto-injector (50 μL loop, Micromeritics Model 725); Hewlett-Packard Model 3354 integration system. Variable wavelength detector (Perkin Elmer LC-75) with auto-control was used for spectral examination of chromatographic peaks using stopped-flow technique. Zorbax 6 μm octadecylsilane (ODS) columns (250 × 4.6 mm id), laboratory-packed columns (250 × 3.2 or 4.6 mm id), and guard columns (50 × 4.6 mm id) with 7–8 μm Zorbax-ODS bulk packing material (DuPont Inc.) were used. Column was maintained at room temperature. Chromatograms were traced on strip chart recorder.

Sample Preparation

Sample treatment.—Prepare internal standard solution of vitamin D_3 of ca 430 IU/mL by pipetting 10 mL vitamin D_3 stock solution into 250 mL low-actinic volumetric flask and diluting to volume with hexane. Pipet 5 mL internal standard solution (~430 IU/mL) into 250 mL screw-cap low-actinic sample bottle and evaporate to dryness under stream of nitrogen at room temperature. Obtain average tablet weight for 20 tablets and grind to fine powder with Spex mill. Accurately weigh sample of powder equivalent to 5 tablets and transfer to sample bottle. Add

40 mL dimethyl sulfoxide to sample, sonicate 30 min at 40–50°C, and shake occasionally. Add 40 mL methanol–water (1 + 1), shake well, and cool to room temperature. To each sample bottle add 80 mL hexane, cap tightly, and shake vigorously 30 min on mechanical shaker. Centrifuge sample bottles at ca 2000 rpm for 5 min. Pipet aliquot (e.g., 50 mL) of clear hexane layer into 125 mL low-actinic boiling flask and evaporate to dryness using stream of nitrogen or rotary evaporator and water bath at or below 30°C. Dissolve residue in 4 mL hexane.

Sample cleanup.—Using a pipet, load 4 mL sample solution into disposable Sep-Pak silica cartridge. Elute unwanted components (vitamins A, E, and excipients) from cartridge by using pipet containing 3 mL 15% (v/v) ethyl acetate–hexane, and discard eluate (3 mL). Elute vitamin D_2 and the internal standard (vitamin D_3) from cartridge by using pipet containing 5 mL 20% (v/v) ethyl acetate–hexane (12). Collect eluate (5 mL) in suitable low-actinic flask and evaporate to dryness under stream of nitrogen. Dissolve residue in 4 mL mobile phase or acetonitrile. Filter through 0.45 μm disposable filter before analysis.

Chromatography and system suitability.—Equilibrate analytical column and guard column with mobile phase until relatively stable baseline is obtained at 265 nm and 0.04 or 0.08 AUFS. Make replicate injections of sample or test solution and check for system suitability (e.g., calculate peak separation, P_s (20)). Select integration parameters carefully so that proper area allocation to each of the fused peaks is reproducible. Maximum value of P_s is unity, which will be the case for baseline resolution between adjacent peaks. Minimum acceptable value of P_s should be 0.8 (i.e., 80% peak separation). If $P_s < 0.8$, improve the chromatographic system. Also, relative standard deviation of area ratios of duplicate injections of same solution should be ≤1%.

Calculations

Calculate concentration of vitamin D_2 or D_3 stock solution as follows:

$$C = W_r \times 40 \times P/100$$

where C = concentration of working standard (IU/mL); W_r = weight of working standard (μg); 40 = units of vitamin D per microgram (IU/μg); P = strength of working standard expressed as a decimal; 100 = dilution factor (mL).

Calculate vitamin D_2 potency as follows:

$$\text{units of vitamin } D_2 \text{ per dosage form} = [(R_a - b)/m] \times (IS/SA)$$

where R_a = area ratio of vitamin D_2 to D_3 in sample; b = intercept of calibration line (area ratio vs concentration ratio); m = slope of same calibration line; IS = amount of internal standard (IU); SA = sample amount (tablets or g).

Results and Discussion

Development of Chromatographic Procedure

Normal phase HPLC cannot separate vitamin D_2 from D_3. The limited solubility of D vitamins in a methanol–water medium is a major factor that results in low column efficiency and/or skewed peaks, and hence, inadequate resolution. Nonaqueous reverse phase HPLC, which is a relatively new approach to the analysis of low polarity samples, has been gaining popularity. The benefit offered by a highly retentive octadecylsilane bonded phase combined with a nonaqueous mobile phase is improved chromatography due to enhanced solubility of nonpolar samples in organic solvents such as methanol, acetonitrile, methylene chloride, and tetrahydrofuran (19).

A preliminary nonaqueous reverse phase HPLC system was tested using a Zorbax-ODS column and methylene chloride–acetonitrile mobile phase. Figure 1 shows the chromatograms of an uncleaned and a cleaned sample preparation in mobile phase. Several features are evident from these chromatograms. First, the sample tested was a multivitamin–mineral preparation and hence its hexane extract was a multicomponent solution. Second, the resolving power of this system displayed a complex elution profile for the sample solution, indicating that unwanted and highly retained or interfering components were in the hexane extract. Figure 1A shows that direct chromatography of a sample extract is not recommended because some unwanted and non-eluted components in the sample will damage the analytical column and lengthen the analysis time, even though resolution is not a problem. Third, the more than adequate resolution of vitamins D_2 and D_3 (Figure 1B) demonstrates the potential of nonaqueous reverse phase HPLC.

Further method development and optimization of the nonaqueous reverse phase HPLC method resulted in selecting a mobile phase of 10% (v/v) methanol in acetonitrile with a Zorbax-ODS column. Figure 2 shows chromatograms of a standard preparation containing ap-

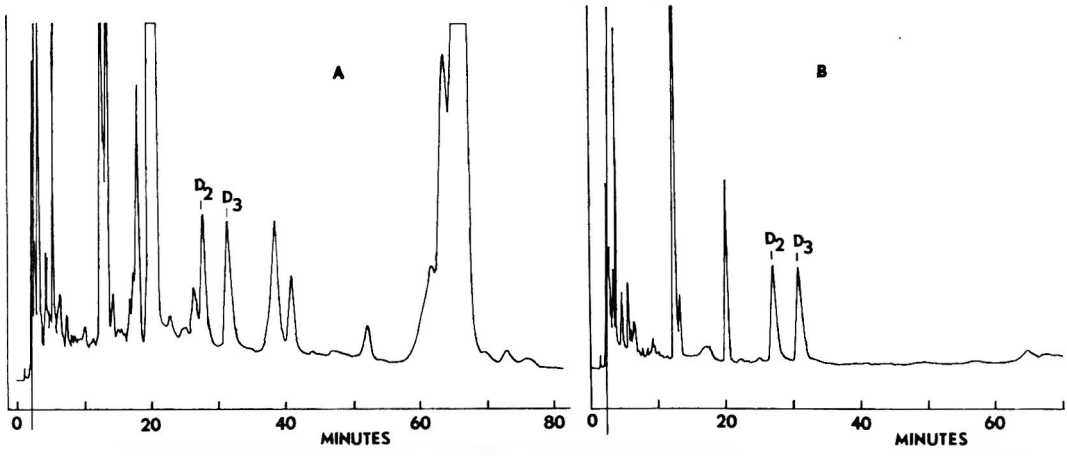

Figure 1. Chromatograms of (0.04 AUFS) of multivitamin-mineral sample preparation: A, before sample cleanup; B, after sample cleanup. D_2, vitamin D_2; D_3, vitamin D_3; identity of other peaks is not known.

proximately equal amounts of vitamins D_2 and D_3, and also a sample preparation. These chromatograms show good resolution and peak shape for the D vitamins.

Method Validation

Specificity.—A method is considered to be specific when the components of interest can be adequately resolved from all other impurities, excipients, and degradation products detected. The nonaqueous reverse phase HPLC system provides adequate specificity in this method: (*1*) *Matching Placebo Assay.*—As shown in Figure 2, baseline resolution of vitamins D_2 and D_3 in both standard and sample can be achieved. The fact that each of the vitamin D_2 and D_3 peaks is free from any hidden, co-eluting excipient peaks was confirmed with a placebo preparation containing all ingredients but vitamin D_2. No peaks detected correspond with those of vitamins D_2 and D_3.

(*2*) *Degradation Products.*—The USP official monograph on ergocalciferol (21) suggests that a standard solution of vitamin D_2 when refluxed in isooctane for 2 h and irradiated under UV light for 1 h should contain ergocalciferol, pre-ergocalciferol and tachysterol. This solution is suitable for a system suitability test. Figure 3 is a representative chromatogram of a vitamin D_2 standard solution (in hexane) refluxed for 3 h

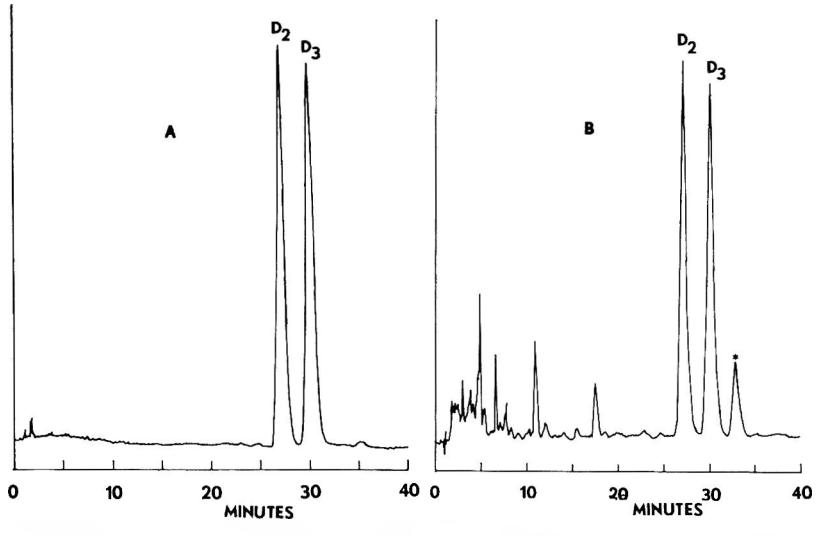

Figure 2. Typical chromatograms (0.08 AUFS) of A, 1:1 standard mixture of vitamins D_2 and D_3; B, multivitamin-mineral sample. D_2, vitamin D_2; D_3, vitamin D_3; *, excipient.

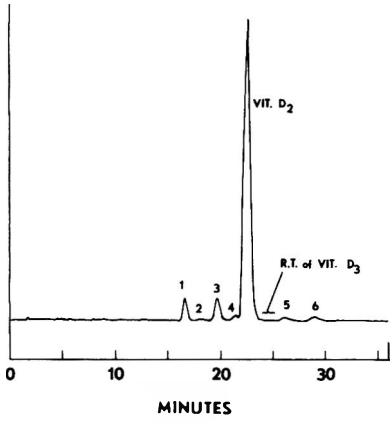

Figure 3. Chromatogram of refluxed and light-degraded standard solution of vitamin D_2. Peaks 1 and 3 are light degradation product (tachysterol) and pre-vitamin D_2, respectively; identity of other peaks was not determined.

followed by 3 h UV irradiation (with a sun lamp) at room temperature. Approximately 6 degradation products were resolved from the main peak of vitamin D_2. The identity of the main peak in the degraded standard was confirmed by comparison of its UV scan with that of a vitamin D_2 peak in a standard. In addition, the retention times of the main peak and that for vitamin D_2 standard were identical.

(3) *Selectivity of Chromatographic System.*—As an additional confirmation of system selectivity, samples with and without vitamin D_3 added were chromatographed with 2 analytical columns in series and a mobile phase of 5% methanol in acetonitrile. This technique is commonly used to detect any hidden, co-eluting peaks that can be resolved from the peaks of interest by increasing system efficiency. The 3 peaks (vitamins D_2, D_3, and excipient) were well resolved with no hint of any unresolved peaks. Also, it was confirmed that no hidden peaks co-eluted in the region where vitamin D_3 eluted.

(4) *Purity of Peaks of Interest.*—Spectral comparison and discrimination techniques were recently introduced (22, 23) into the field of HPLC to aid chromatographers in further characterizing species eluting from an analytical column. These techniques are based on the corrected absorbance values at several (up to 9) wavelengths of a stopped-flow portion of the component of interest. The stopped-flow portion could be any part of a chromatographic peak. It is suggested that if the value of spectral discrimination ratio (D) is 1.5 or less, then there is strong evidence that the 2 spectra correspond to the same component. This same argument applies to the different (beginning, middle, and end) portions of a chromatographic peak and thus helps confirm the homogeneity of the peak of interest. The spectral discrimination technique was used to test the purity and homogeneity of the vitamin D_2 and D_3 peaks in the chromatograms of a reference standard and an aged stability sample. The peaks in the sample were spectrally identical to the corresponding vitamin D_2 and D_3 peaks in the standard (all D values were 1.3 or less).

Accuracy.—To determine the accuracy of the method, powdered placebos were spiked at 3 different levels, similar to the 3 standards, and were assayed for samples. Table 2 shows recovery data obtained on 2 separate days and their corresponding re-assay values after one day storage under refrigeration. Recovery ranges from 98.4 to 102.8% and from 97.2 to 101.0% on 2 different days, with averages of 100.6 and 98.9%, respectively. Recovery is judged to be quantitative and adequate.

Precision.—The reproducibility of the method

Table 2. Recovery of vitamin D_2 added to placebos at 3 levels

			Found, IU		Recovery, %	
Day	Sample	Added, IU	Detn 1	Detn 2[a]	Detn 1	Detn 2
1	Placebo	none	none	—	—	—
	Spiked placebo	1068.0	1076.3	1091.1	100.8	102.2
	Spiked placebo	2136.0	2143.0	2196.0	100.3	102.8
	Spiked placebo	2136.0	2194.8	2173.4	102.8	101.8
	Spiked placebo	3204.0	3151.3	3220.5	98.4	100.5
				Mean	100.6	101.8
2	Spiked placebo	981.9	991.8	1022.3	101.0	104.1
	Spiked placebo	1963.8	1932.8	1963.9	98.4	100.0
	Spiked placebo	1963.8	1908.9	2034.3	97.2	103.6
	Spiked placebo	2945.8	2912.0	3005.9	98.9	102.0
				Mean	98.9	102.4

[a] Detn 2 data were obtained from standards and samples re-assayed after 1 day storage under refrigeration in the dark.

Table 3. Long term precision data on vitamin D_2 in multivitamin tablets [a] stored at 4°C

Date of assay	Found,[b] IU/tab.	CV, % (n = 2)	% Input[c]
8/15/80	620	1.50	100.2
8/26/80	604	0.72	97.5
9/2/80	619	2.72	100.0
10/14/80	592	2.46	95.6
11/21/80	595	1.26	96.1
12/10/80	602	3.67	97.3
1/2/81	588	0.10	95.0
Overall av.	603	2.09	97.4

[a] Super Paramette, Batch QWE-YG, Ayerst Laboratories Inc., Montreal, Quebec, Canada.

[b] Three different columns of Zorbax-ODS were used by 2 analysts over the period indicated.

[c] Input = 619 IU/tablet (initial). Sample kept one month at room temperature before precision study.

was assessed by determining instrumental, intra-day, and inter-day precision. For this method the following was established:

Precision	*Typical CV (%)*
Instrumental	0.27–0.34 (for 10 injections of 1 sample in 1 day)
Intra-day	0.64–2.38 (for duplicate samples assayed on same day)
Inter-day	1.75 (separate weights of the same sample pool assayed over 2 separate days)

The long term precision of the method is based on the repeated assay of one or more batches of multivitamin–mineral tablets stored at 4°C over a period of several months. It is assumed that these samples do not change during this time at 4°C.

The long term precision data for a batch of multivitamin-mineral tablets are shown in Table 3. The following points should be noted: (*1*) There appears to be a gradual but slight loss in the strength of the tablets for the batch monitored. This suggests that storage conditions at 4°C alone is not adequate for keeping multivitamin-mineral tablets unchanged in vitamin D_2 content. (2) The CV for the long-term precision data is 2.09%. This includes other sources of variation such as tablet-to-tablet uniformity, aging effects, storage conditions, columns, and analysts.

Linearity.—Three-level calibration curves are recommended for this method. The concentration levels (approximately 100–600 IU vitamin D_2/mL) chosen should bracket all samples being assayed.

The linearity (expressed as standard deviation of % Y-axis or $s_{y\cdot x}$ (24)) is a measure of the error of the calibration curve. A smaller $s_{y\cdot x}$ value indicates less error and hence more reliable quantitation. Table 4 lists all linearity data. The small $s_{y\cdot x}$ values strongly support the use of vitamin D_3 as an internal standard. It is recommended that the error estimate ($s_{y\cdot x}$) be within 2%.

Table 4. Standard error data of typical 3-level calibration curves

Date	$s_{y\cdot x}$%	No. points on curve
8/26/80	1.12	6
8/27/80	1.21	6
9/ 2/80	0.52	5
9/ 3/80	0.49	3
10/30/80	0.91	6
10/31/80	0.31	6
11/19/80	0.80	6
11/21/80	0.40	5
11/24/80	0.79	6
11/26/80	1.04	6
12/10/80	1.10	10
1/ 2/81	0.25	6

Sensitivity.—With a sample injection size of 50 μL and detection at 265 nm and 0.04 AUFS, the detection limits for vitamins D_2 and D_3 are 8 and 9 IU/mL, respectively, based on a peak 1% of full scale. This strength method is used for tablets containing 300–600 IU vitamin D/tablet (i.e., sample concentration of about 310 IU/mL) and is considered adequate for this purpose.

Comparison of Two Disposable Cleanup Cartridges

Two types of disposable silica cartridges were compared for sample cleanup. Sep-Pak cartridges were used throughout the development process and were easy to use. Bond-Elut silica cartridges (1 mL capacity) were evaluated against Sep-Pak for cleanup efficiency. Figure 4 shows representative chromatograms of portions of the same sample work-up, eluted through the 2 different silica cartridges. There are minor differences between the chromatograms but both are acceptably free of contamination.

Summary

A method has been developed for the determination of vitamin D_2 in multivitamin dosage forms. The internal standard, vitamin D_3, is very similar to vitamin D_2 and they elute together from silica gel columns in normal phase HPLC. The fact that they cannot be resolved with normal phase HPLC was a favorable condition for the sample cleanup step using silica cartridges.

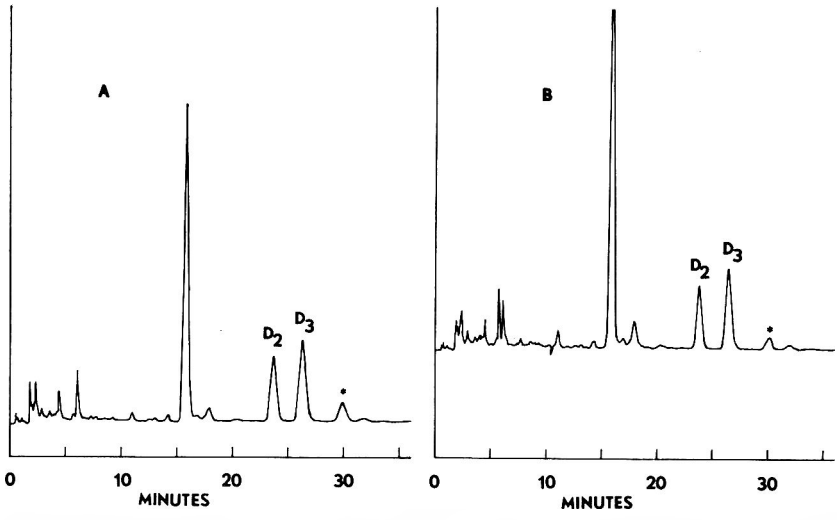

Figure 4. Typical chromatograms of a sample cleaned by using A, Sep-Pak; B, Bond-Elut silica. For B: cleanup solution, 0.5 mL ethyl acetate-hexane (10 + 90); eluting solution, 3.0 mL ethyl acetate-hexane (20 + 80).

A real source of concern in the determination of vitamin D_2 is the neglect of the slow reversible isomerization, vitamin D ⇌ pre-vitamin D (25). To account for the vitamin D-pre-vitamin D equilibrium in samples without measuring the pre-vitamin D, several pharmaceutical companies recommended that both the reference standard and the sample be treated at 80°C for 2½ h (26). The use of vitamin D_3 as the internal standard for the determination of vitamin D_2 content in multivitamin preparations is recommended since both vitamins D_2 and D_3 have received identical treatment from the very first step of sample preparation. It was shown that the isomerization rates of vitamins D_2 and D_3 were equal (25), therefore the ratio of vitamin D_2 to D_3 at any given temperature should be constant.

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FERTILIZERS

Atomic Absorption Spectroscopic Analysis of Aluminum Sulfate-Type Soil Acidifiers: Mini-Collaborative Study

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Commercial brand aluminum sulfate soil acidifiers were subjected to a simple water extraction and analyzed for aluminum content by applying the principles of AOAC method 8.023. The study was designed to follow Youden's procedure for closely matched pairs and yielded results in close agreement. Statistical analysis, using 98% confidence limits, showed no outliers among analyses or laboratories. The combined S_r value was 0.1702, indicating satisfactory within-laboratory precision. The method has been adopted official first action.

The rationale behind the water extraction of aluminum sulfate-type soil acidifiers is that it is similar to the solvent action in the soil that would produce the acidifying action.

Eight collaborators were contacted, all of whom completed the study. The number of collaborators is small because the purpose of this study was to test the feasibility of applying the principles of an established method (AOAC 8.023, ref. 1) to a different product and also because it was difficult to locate laboratories having suitable atomic absorption spectrophotometers that could burn N_2O and/or having an aluminum lamp.

All collaborators received aluminum pellets for preparation of their stock standard, a quantity of aluminum sulfate reagent to be used for familiarizing themselves with the method, and 8 samples (4 matched pairs) for analysis. The matched samples pairs consisted of the following:

Pair 1: Samples A and F = commercial brand aluminum sulfate soil acidifier (Al guar. = 9%)

Pair 2: Samples B and E = commercial brand aluminum sulfate soil acidifier (Al guar. = 9%)

Pair 3: Samples C and H = 50% commercial brand aluminum sulfate soil acidifier (same as A and F) plus 50% commercial fertilizer containing the following:

Element	*Guarantee, %*	*Found, %*
N	20	19.7
P_2O_5	10	19.7
K_2O	5	6.9
S	6	5.8
Fe	4	4.1

Pair 4: Samples D and G = 50% commercial brand aluminum sulfate soil acidifier (same as B and E) plus 50% Celite.

Collaborators were instructed to run single determinations on Samples A, B, C, and D on one day, and single determinations on Samples E, F, G, and H on another day.

Aluminum in Aluminum Sulfate-type Soil Acidifiers

Atomic Absorption Spectrophotometric Method

Official First Action

2.D23 ***Apparatus and Reagents***

(**a**) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 303, or equiv. See **8.023** for typical operating parameters.

(**b**) *Diluting soln.*—Add 20 mL H_2SO_4 and 2.5 g NaCl to 500 mL H_2O. Dil. to 1 L with H_2O.

(**c**) *Aluminum std solns.*—(*1*) *Stock soln.*—1 mg Al/mL (1000 ppm). Accurately weigh 1.000 g pure Al and dissolve in ca 25 mL HCl. Evap. almost to dryness, add 500 mL H_2O, 20 mL H_2SO_4, and 2.5 g NaCl, and dil. to 1 L with H_2O. (*2*) *Working solns.*—Dil. aliquots of stock soln with dilg soln, (**b**), to make 4 std solns within range 50–250 ppm.

2.D24 ***Preparation of Sample***

Accurately weigh ca 1 g sample into 500 mL screw-cap erlenmeyer, add 250 mL H_2O, and

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shake on wrist-action shaker ca 15 min. Quant. transfer to 500 mL vol. flask and dil. to vol. with H_2O. Filter thru Whatman No. 2 paper. Dil. aliquot as necessary (diln factor = F) with dilg soln, (**b**), for concn range 50–150 ppm.

2.D25 *Determination*

Set up app. as shown in Table **8:01,** or use previously established optimum settings for app. Zero app. while aspirating dilg soln, (**b**). Det. A of std solns within 50–150 ppm range, alternating with sample soln readings. Flush burner with dilg soln, (**b**), and check zero point between readings.

Det. Al content from std curve of A against μg Al/mL.

$$\% \text{ Al} = (\mu\text{g Al/mL}) \times F \times (500/\text{g sample}) \times 10^{-4}$$

Results and Discussion

The study was designed to follow Youden's procedure for closely matched pairs (2). Samples designated F, E, H, and G were blind duplicates of A, B, C, and D, respectively.

The results from Pair 3 (C/H) and Pair 4 (D/G) had large standard deviations and coefficients of variation. Both the author and the statistical consultant agreed that this could be attributed to variation among 1 g samples weighed from mixtures such as these samples were. Because this problem is separate from the purpose of the study, the results from these pairs were omitted.

The results from Pair 1 (A/F) and Pair 2 (B/E) were in close agreement; the collaborators' results for these pairs are shown in Table 1.

Table 2. Summary statistics for Sample Pairs 1 and 2

Statistic	Sample Pair 1	Sample Pair 2	Sample Pairs 1 & 2 combined
N	18	18	36
$\bar{X}$	8.317	8.196	8.256
S_r	0.2017	0.1313	0.1702
S_b	0.1927	0.1896	0.1912
S_d	0.2789	0.2306	0.2559
CV, %	3.35	2.81	3.09

Matched pair calculations were made by Youden's method; 98% confidence limits were applied to the sums of paired results to identify outliers. There were no outliers among analyses or among the R1 − R2 differences. The range test was used to show that there were no outlier laboratories.

The summary statistics for Pair 1 and Pair 2 are shown in Table 2. Within-laboratory deviation (repeatability), between-laboratory deviation (bias), and overall standard deviations are designated by the symbols S_r, S_b, and S_d, respectively; S_d is defined as $\sqrt{S_r^2 + S_b^2}$. These 3 deviations were determined, and the S_d value was used to calculate the coefficient of variation (CV).

With regard to comments and suggestions from collaborators, three recommended that all samples be diluted with diluting solution regardless of the aluminum concentration, and that the sample and standard solutions contain the same or nearly the same concentrations of H_2SO_4 and NaCl. Two collaborators felt that some of the samples, especially C, D, G, and H, were not ground sufficiently to obtain a homogeneous mixture. One collaborator commented that

Table 1. Collaborator results [a] (%) for determination of aluminum from aluminum sulfate-type soil acidifiers

	Aluminum, %			
	Sample Pair 1		Sample Pair 2	
Coll.	A	F	B	E
1	8.39	8.41	8.37	8.40
2	8.14	7.92	8.03	7.92
3	8.50	8.49	8.34	8.32
4	8.38	8.45	8.10	8.20
5	8.76	8.50	8.40	8.40
6	8.05	7.77	8.08	7.61
7	8.19	8.14	8.00	7.97
8	8.20	8.91	8.31	8.56
9	8.33	8.17	8.23	8.28
	N = 18		N = 18	
	$\bar{X}$ = 8.317		$\bar{X}$ = 8.196	

[a] Including author's results.

Samples D and G caused an accumulation of materials on the burner head which resulted in partially clogging it.

Recommendation

It is recommended that this method for the analysis of aluminum from aluminum sulfate-type soil acidifiers be adopted official first action.

Acknowledgments

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Argon Plasma Emission Spectrometry of Uranium in Phosphatic Materials

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A method is described for the determination of uranium by using a dc argon plasma emission spectrometer. Problems and interferences found in the method previously reported are overcome by using an improved sample pretreatment. A new synergistic system, 2% oxalic acid adjusted to pH 4.5 with triethanolamine, is used to quantitatively back-extract uranium from trioctylphosphine oxide in cyclohexane. This improved method yields excellent results for >1 ppm uranium in nitric and hydrochloric acid solutions of phosphate rocks, phosphoric acids, phosphate fertilizers, phosphogypsums, organic phosphates, and waste solutions. Recovery data are given for synthetic and certified standards.

A previous study (1) has shown the feasibility of determining uranium in wet-process phosphoric acid and phosphate rock by using argon plasma emission spectrometry (APES) after extraction of uranium with trioctylphosphine oxide (TOPO). Further research on uranium recovery and on the fate of uranium in phosphoric acid and fertilizer processes has introduced an even greater need for speed, accuracy, and precision in the determination of uranium at low concentrations in a variety of phosphatic materials.

Although the method previously reported (1) can be performed on these types of samples, it suffers from relatively low limits of extractability and less than desirable tolerance of certain impurities, and involves the use of a difficult and time-consuming acid decomposition of organic material.

This new method, although similar in principle, includes several improvements: (*1*) use of a different acid matrix in the extraction of uranium by TOPO in cyclohexane; (2) use of ascorbic acid as a reducing agent to eliminate iron as an interference in the extraction and post-extraction steps; (*3*) use of a new synergistic system for the quantitative back-extraction or "stripping" of uranium from TOPO in cyclohexane, thereby eliminating acid decomposition of TOPO; (*4*) determination of uranium in the stripping solution by APES.

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METHOD

Apparatus and Reagents

(**a**) *Spectrometer.*—Spectraspan III dc argon plasma (Spectrametrics, Inc., 204 Andover St, Andover, MA 02840).

(**b**) *Uranium standard solution.*—1000 μg U/mL. Dissolve 1.1793 g NBS No. 958 (U_3O_8) in 20 mL HNO_3 and dilute to 1 L.

(c) *Trioctylphosphine oxide (TOPO).*—0.075M. Dissolve 29 g TOPO in cyclohexane and dilute to 1 L with cyclohexane.

(**d**) *Cyclohexane.*—Technical grade.

(**e**) *Triethanolamine (TEA).*—Reagent grade.

(**f**) *Oxalic acid (4%)-TEA solution.*—Weigh 40 g oxalic acid dihydrate into 1 L beaker, dissolve in ca 800 mL water, and adjust pH to 4.5 with TEA. Transfer solution to 1 L volumetric flask and dilute to volume with water.

(**g**) *Stripping solution.*—Dilute oxalic acid (4%)-TEA solution with equal volume of water.

(**h**) *Ascorbic acid solution.*—Dissolve 100 g ascorbic acid in water and dilute to 1 L. Prepare monthly.

Sample Preparation

(**a**) *Phosphoric acid and other acid-soluble phosphatic materials.*—Weigh appropriate size sample into a beaker. (Quantity of sample is governed by concentrations of uranium, phosphorus, and acid. Generally, 1-5 g is sufficient.) Add a few drops of H_2O_2 and digest in HCl ca 10 min. If sample contains noticeable amount of organic material, add 2 mL H_2SO_4 instead of H_2O_2 and HCl, heat to light fumes of H_2SO_4, and add HNO_3 dropwise until organic material is completely oxidized. Evaporate to dense fumes of H_2SO_4, cool, add ca 50 mL water, and boil 10 min.

(**b**) *Organic liquids.*—Weigh 1-5 g sample into a tall, lipped beaker or Erlenmeyer flask. Evaporate to near dryness, add 2 mL H_2SO_4, and, while heating, add HNO_3 dropwise until organic material is completely oxidized. Evaporate to dense fumes of H_2SO_4, cool, add ca 50 mL water,

and boil 10 min. If necessary, some HCl may be added to help dissolve solids.

(c) *Phosphate rock and gypsum filter cakes.*— Weigh ≤ 2 g sample into beaker, add 25 ml HCl, and digest 10–15 min. Dilute to ca 80 mL with water, add a few drops of H_2O_2, and boil to dissolve solids and decompose H_2O_2. Cool, filter, transfer residue to Pt crucible, and ignite. Cool, add 1 mL H_2SO_4, and 2–3 mL portions of HF until silica evolves. Continue heating to fumes of H_2SO_4. Cool and combine with filtrate. Any insoluble residue remaining from HF-H_2SO_4 digestion can be fused with pyrosulfate, dissolved in HCl, and added back to filtrate.

Extraction of Uranium

Transfer entire digested sample or aliquot containing ≤2500 μg U, ≤4.0 g P_2O_5, and ≤4 mL H_2SO_4 to 125 mL separatory funnel. Adjust HCl content to ca 20–25 mL, dilute to ca 100 mL with water, add 4 mL of ascorbic acid solution, and mix. Let yellow of Fe(III) fade. If color does not fade, add additional 2 mL ascorbic acid solution and mix. Add 10 mL TOPO and shake well for 1 min. Set aside and let layers completely separate. Thoroughly drain aqueous layer and discard. Pipet 25 mL stripping solution into separatory funnel, shake well for 1.5 min, and set aside to let layers completely separate. Discard first few milliliters, then collect remaining aqueous layer for APES determination of uranium. Discard TOPO layer.

If some alternative sample preparation must be used which results in the presence of $HClO_4$ or HNO_3 in final digests, use following extraction: Transfer entire digested sample or aliquot containing ≤1000 μg U, ≤1.0 g P_2O_5, and ≤5.0 mL $HClO_4$ to a 125 mL separatory funnel. Adjust HNO_3 content to 6 mL and mix, dilute to ca 100 mL with water, and proceed as before, beginning with addition of TOPO.

Determination of Uranium

From uranium standard solution, prepare minimum of 5 working standards containing 0–100 μg U/mL. Match standards with samples, using oxalic acid (4%)–TEA solution. Compare samples and standards with APES system at 424.17 nm.

Results and Discussion

The described method, like that of Woodis et al. (1), relies on the extractability of uranium from mineral acid solution. TOPO in cyclohexane is the reagent of choice for this extraction. However, the method of Woodis et al. specifies extraction of uranium from a primarily nitric acid matrix. Although that matrix favors quantitative extraction of uranium by TOPO in cyclohexane as $UO_2(NO_3)_2 \cdot 2(TOPO)$, uranium extractability is limited by the co-extraction of nitric acid in TOPO (2). The extraction coefficient of uranium is decreased by the presence of phosphate, sulfate, and perchlorates, as well as excess amounts of nitric acid.

Table 1. Permissible conditions for quantitative uranium extraction

Parameter	HCl matrix	HNO_3 matrix
U, μg	≤2500	≤1000
P_2O_5, g	≤4.0	≤1.0
H_2SO_4,[a] mL	≤4.0	≤0.5
HCl,[a] mL	20–25	≤10
$HClO_4$,[a] mL	0.0	≤5.0
HNO_3,[a] mL	0.0	6.0

[a] As concentrated reagent acid.

Studies indicate that TOPO in cyclohexane can quantitatively extract up to 1000 μg uranium from a nitric acid matrix containing relatively small amounts of sulfate, perchlorate, and phosphate (Table 1). However, by using a hydrochloric acid matrix, up to 2500 μg uranium can be quantitatively extracted in the presence of relatively more phosphate and sulfate (Table 1). Neither nitric acid nor perchloric acid can be tolerated in any significant amount in the extraction from this matrix. However, sample preparation can usually be adjusted to avoid these acids. Significant amounts of Fe(III) also cannot be tolerated in the extraction from the hydrochloric acid matrix. Ferric iron in the presence of chloride is co-extracted with uranium by TOPO, thus decreasing uranium extractability (2). However, Fe(II) is not extracted by TOPO.

Tests of several reducing agents revealed that small amounts of ascorbic acid (0.4%) in the initial aqueous phase reduced Fe(III) to Fe(II). This allows quantitative extraction of uranium, and also effectively eliminates iron as an interference in post-extraction operations.

In addition to benefits to the initial extraction of uranium, the hydrochloric acid matrix also facilitates the back-extraction of uranium from TOPO. The uranium from the initial adduct, $UO_2Cl_2 \cdot 2(TOPO)$, is more easily stripped than is $UO_2(NO_3)_2 \cdot 2(TOPO)$.

A series of tests was carried out to find a good stripping solution for the back-extraction of uranium from TOPO. Several stripping solutions were tried with varying degrees of success,

Table 2. APES determination of uranium (μg/g) in certified phosphate rock

Sample	Certified value	Found, av.[a]	SD
NBS 120B	128.4 ± 0.5	130.25	1.50
NBL 1A	153 ± 2	155.75	1.86

[a] Twelve determinations.

including ammonium carbonate, sulfuric acid, ammonium oxalate, oxalic acid, oxalic acid (2%)-triethanolamine (TEA), and others. Problems were encountered with all but two, oxalic acid (2%)-TEA and sulfuric acid. Of these, oxalic acid (2%)-TEA was the better, because it was possible to back-extract with a single, known volume of stripping solution and thereby eliminate subsequent dilutions. Sulfuric acid is also more hazardous and difficult to handle. The use of oxalic acid (2%)-TEA is a new synergistic system for the quantitative extraction of uranium from TOPO in cyclohexane.

An aqueous solution of oxalic acid (2%) that has been adjusted to pH 4.5 with triethanolamine (TEA) can be used for quantitative back-extraction of uranium from TOPO in cyclohexane. It successfully strips uranium from both adducts, $UO_2(NO_3)_2 \cdot 2(TOPO)$ and $UO_2Cl_2 \cdot 2(TOPO)$. Since the back-extraction is quantitative for either initial matrix, hydrochloric or nitric acid, it can be used for either sample preparation.

This back-extraction replaces the TOPO destruction step used in the method of Woodis et al., which specifies extraction of uranium, followed by evaporation of cyclohexane and ternary acid destruction of the residual TOPO. This results in a sulfuric acid solution of uranium which is made to volume and the uranium concentration measured by APES. This has been a useful method yielding satisfactory results with only a few problems. However, ternary acid destruction of the residual TOPO is time consuming, can yield final solutions of inconsistent matrices, and is potentially dangerous.

Oxalic acid (2%)-TEA back-extraction saves time, eliminates a hazard, and yields a known matrix for APES determination.

To check the accuracy and precision of the method, 2 certified phosphate rocks were analyzed independently 12 times. One of the rocks was supplied by the National Bureau of Standards and is designated 120B. The other rock was obtained from the Department of Energy, New Brunswick Laboratory, and is designated NBL 1A. The average results were acceptably close to the certified values; the precision, as measured by the reported standard deviations, was acceptable (Table 2).

Table 3. Recovery (μg/g) of known amounts of uranium added to phosphatic materials

Material	Added	Found[a]	Rec., %
Triple superphosphate	0	210	—
	50	263	106
	100	311	101
	150	365	103
Diammonium phosphate	0	231	—
	50	281	100
	100	335	104
	150	389	105
Suspension, 9-32-0	0	85	—
	50	136	102
	100	188	103
	150	236	101
Phosphoric acid	0	38	—
	50	89	102
	100	139	101
	150	189	101
Gypsum	0	65	—
	50	115	100
	100	165	100
	150	213	99

[a] Each value is the average of 3 determinations.

A completely independent method was not available to compare with the proposed method. To demonstrate the analysis of products, 5 phosphatic materials were selected, and each was analyzed in triplicate by the proposed method. Each material was then spiked with 3 levels of known uranium concentration and re-analyzed. Recoveries (Table 3) were calculated without regard to the quantity of uranium present in the starting material. Recoveries at all levels tested were excellent, indicating that the method, as applied to the materials tested, was very reliable.

This study shows that a new procedure for the determination of uranium by dc argon plasma emission spectrometry coupled with TOPO extraction and a back-extraction can improve the speed and reliability of determining uranium in a variety of phosphatic materials.

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Atomic Absorption Spectrophotometric Determination of Chelated Iron in Iron Chelate Concentrates: Collaborative Study

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An atomic absorption spectrophotometric (AAS) method for determining chelated iron in iron chelate concentrates was collaboratively studied by 13 laboratories. Nonchelated iron was selectively precipitated as ferric hydroxide from an aqueous solution of the sample by adjusting to pH 8.5 with sodium hydroxide. A filtered portion of the sample solution was diluted with 0.5N HCl, and the iron was determined by AAS using standards containing disodium EDTA. Six pairs of iron chelate materials (12 samples) were analyzed and the average coefficients of variation were iron EDTA 3.52%, iron HEDTA 4.16%, iron DTPA 3.55%, iron EDDHA 3.52%, iron citrate 2.86%, and iron DPS 8.47%. The method has been adopted official first action.

Chelated iron is currently used as a micronutrient source for lawns, gardens, and commercial crops where soil pH, high phosphate, or other factors limit the solubility of inorganic iron salts. Soluble iron chelates are usually applied along with a mixed fertilizer or as a solution sprayed directly on foliage. A method is therefore needed to distinguish chelated and nonchelated forms of iron. Precipitation of nonchelated iron as the hydrous oxide or phosphate has been suggested as a means of distinguishing the chelated forms of iron (C. E. Jones, Colorado Dept of Agriculture, 1975; J. D. Warren, Florida Dept of Agriculture and Consumer Services, 1976). The Florida Department of Agriculture and Consumer Services has developed and studied a procedure which is based on the precipitation of nonchelated iron at pH 8.5 and the determination of soluble chelated iron by atomic absorption spectrophotometry (AAS) (J. D. Warren and C. L. Jones, 1980). This procedure, with some modification, was used in the collaborative study.

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The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

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Before subjecting the proposed method to a collaborative study, several variables in the procedure were tested for ruggedness (1). The following 5 factors were tested: volume alcohol added, A = 1 mL, a = 5 mL; volume H_2O_2 added, B = 2 drops, b = 5 drops; pH adjustment, C = 8.5, c = 9.5; time of standing after pH adjustment, D = 10 min, d = 30 min; normality of HCl in final solution, E = 0.5N, e = 0.2N. The effects of varying these conditions are listed in Table 1. Generally, the factors have effects of about 1% or less on the analytical result with respect to the total iron present. Significant error is observed only for the addition of excess alcohol to samples of ferric citrate and iron DPS, and the use of a lower concentration of HCl in the final solution of iron DPS. The use of alcohol with ferric citrate and iron DPS is unnecessary because these products are formulated as liquids and need no wetting.

Collaborative Study

Twelve samples of iron chelates were distributed to 13 laboratories. All samples used in the study, with the exception of iron citrate, were prepared from commercial samples of iron chelates. The iron citrate samples were prepared from ferric citrate obtained from the J. T. Baker Chemical Co. Two samples were prepared from each of the 6 iron chelates with the following approximate guarantees: Samples 10 and 12, iron ethylenediaminetetraacetate (EDTA), 13% Fe; Samples 2 and 9, iron hydroxyethylethylenediaminetriacetate (HEDTA), 5% Fe; Samples 3 and 7, iron diethylenetriaminepentaacetate (DTPA), 10% Fe; Samples 6 and 11, iron ethylenediaminedi-*o*-hydroxyphenylacetate (EDDHA), 6% Fe; Samples 5 and 8, iron citrate, 1.4% Fe; Samples 1 and 4, iron *N,N*-bis(2-hydroxy-5-sulfobenzyl)glycine (DPS), 4% Fe. Paired samples for each of the chelates were prepared by diluting liquid chelates with water and adding Kaolin to powdered chelates. Although collaborators were not asked to perform

Table 1. Differences resulting from change of method conditions

	$X - x$, % Fe					
Factor[a]	Fe EDTA 13% Fe	Fe HEDTA 5% Fe	Fe EDDHA 6% Fe	Fe DTPA 10% Fe	Fe Citrate 1.4% Fe	Fe DPS 4% Fe
Alcohol	−0.030%	0.050%	−0.065%	0.008%	0.035%	0.085%
H_2O_2	−0.100	−0.015	0.000	0.028	0.000	0.060
pH	0.025	0.015	−0.035	0.018	0.010	0.030
Time	−0.005	0.000	−0.045	−0.013	−0.005	−0.050
HCl	−0.030	−0.050	−0.080	−0.043	−0.015	0.29

[a] See text for explanation of factors.

practice analyses, they were informed that sufficient sample material was available to do so. Collaborators were asked to perform single determinations on each sample.

Chelated Iron in Iron Chelate Concentrates

Atomic Absorption Spectrophotometric Method

Official First Action

(Applicable to Fe ethylenediaminetetraacetate (EDTA), Fe hydroxyethylethylenediaminetriacetate (HEDTA), Fe diethylenetriaminepentaacetate (DTPA), Fe ethylenediaminedi-*o*-hydroxyphenylacetate (EDDHA), Fe *N,N*-bis(2-hydroxy-5-sulfobenzyl)glycine (DPS), and Fe citrate. Not applicable to mixed fertilizers, or to samples contg non-chelated metals other than Fe.)

2.D16 ***Principle***

Sample is dissolved in H_2O, and non-chelated Fe is pptd as $FeOH_3$ at pH 8.5 and removed. Chelated Fe is detd by AAS, using std solns contg Na_2H_2EDTA.

2.D17 ***Apparatus and Reagents***

(a) *Sodium hydroxide soln.*—0.5N. Dissolve 20 g NaOH in H_2O and dil. to 1 L.

(b) *Disodium EDTA soln.*—0.66%. Dissolve 0.73 g $Na_2H_2EDTA.2H_2O$ in H_2O) and dil. to 100 mL.

(c) *Iron std solns.*—(*1*) *Stock soln.*—1000 μg Fe/mL. Dissolve 1.000 g pure Fe wire in ca 30 mL 6N HCl with boiling. Dil. to 1 L. (*2*) *Intermediate soln.*—100 μg Fe/mL. Pipet 10 mL Fe stock soln and 10 mL Na_2H_2EDTA soln into 100 mL vol. flask and dil. to vol. with H_2O. (*3*) *Working solns.*—Dil. aliquots of intermediate soln with 0.5N HCl to make ≥4 std solns within range of detn (2–20 μg Fe/mL).

(d) *Atomic absorption spectrophotometer.*—With air–C_2H_2 flame. See **2.109(a).**

2.D18 ***Determination***

Weigh sample contg ca 40 mg Fe into 200 mL tall-form beaker. Wet with 2–3 drops of alcohol and dissolve in 100 mL H_2O. Add 4 drops of 30% H_2O_2, mix and adjust pH of soln to 8.5 with 0.5N NaOH. If pH drifts above 8.8, discard soln and repeat analysis. Transfer soln to 200 mL vol. flask, dil. to vol. with H_2O, and mix. Filter soln thru quant. paper. Pipet 10 mL filtrate into 200 mL vol. flask and dil. to vol. with 0.5N HCl. Measure *A* of solns, using lean air-C_2H_2 flame as in **2.112** and det. concn of Fe in sample (μg Fe/mL) from either calibration curve or digital concn readout. In same manner, det. Fe blank on all reagents used.

$$\% \text{ Chelated iron} = (\mu g \text{ Fe/mL in sample} - \mu g \text{ Fe/mL in blank}) \times 0.4/g \text{ sample}$$

Results and Discussion

Results from the 13 collaborating laboratories are shown in Table 2. The statistical analysis of results was carried out using the procedures described by Youden and Steiner (1) for a set of closely matched sample pairs. Results were subjected to Youden's ranking test (1) to determine outlying laboratories and the ranks are listed in Table 2. The allowable score limits for 13 laboratories and 12 samples are 43 to 125 at the 99% confidence limits (2). Three laboratories, two scoring high and one low, fell into the 1% 2-tail limits. Since discussions with these laboratories failed to reveal any factors which might have been responsible for the apparent systematic error, the data were included in all statistical calculations. The result obtained by Collaborator 12 for Sample 10 was discarded because the

Table 2. Collaborative results for determination of chelated iron (%)

	Samples												Total
Coll.	1	2	3	4	5	6	7	8	9	10	11	12	rank
1	4.02	4.81	9.69	3.74	1.42	6.09	9.16	1.27	4.89	12.00	6.27	12.61	116.5
2	3.5	4.6	9.2	3.2	1.4	5.9	9.1	1.3	4.7	11.6	6.2	12.2	144.5
3	4.11	4.73	9.30	3.76	1.45	5.91	9.24	1.32	4.87	12.13	6.27	11.88	120
4	4.12	4.82	9.87	3.88	1.51	6.54	9.92	1.36	5.16	12.16	6.60	13.00	56
5	3.32	4.79	9.94	3.04	1.47	5.98	9.72	1.34	4.77	12.40	6.24	12.35	105.5
6	3.81	4.96	10.00	4.05	1.47	6.33	9.86	1.34	5.13	12.59	6.53	13.03	55.5
7	4.12	4.81	9.82	4.00	1.48	5.97	9.67	1.31	5.01	12.30	6.34	12.97	89
8	3.70	5.11	10.18	3.59	1.50	6.59	10.08	1.36	5.37	12.49	6.91	13.07	44.5
9	4.26	4.96	10.10	4.14	1.50	6.23	10.03	1.41	5.32	12.96	6.45	13.75	31.5
10	4.22	4.82	9.87	4.00	1.46	6.13	9.69	1.32	4.98	12.37	6.34	12.76	82.5
11	4.39	4.92	9.94	4.10	1.50	6.34	9.71	1.34	5.12	12.56	6.52	13.22	46.5
12	3.97	4.57	9.37	3.84	1.42	6.18	8.87	1.30	4.77	11.36[b]	6.12	12.17	131.5
13	4.30	4.84	9.68	3.94	1.37	6.33	9.57	1.21[a]	5.62	12.70	7.24[a]	13.31	68.5
Av.	3.99	4.83	9.77	3.79	1.46	6.19	9.59	1.33	5.06	12.36	6.40	12.79	
SD	0.321	0.145	0.307	0.337	0.0436	0.226	0.380	0.0363	0.269	0.356	0.217	0.531	
CV, %	8.05	3.00	3.14	8.89	2.99	3.65	3.96	2.73	5.32	2.88	3.39	4.15	

[a] Result rejected as outlier, using Dixon test at 95% confidence limit (3).
[b] Result not included in statistical analysis, sample pH adjusted too high.

Table 3. Statistical analysis of sample pairs

Iron chelate	Pair	S_d	S_r	S_b	*F*-ratio	DF
Fe DPS	1,4	0.4517	0.1119	0.3094	16.29	12
Fe HEDTA	2,9	0.2729	0.1375	0.1667	3.94	12
Fe DTPA	3,7	0.4739	0.1170	0.3247	16.41	12
Fe Citrate	5,8	0.0487	0.0164	0.0324	8.82	11
Fe EDDHA	6,11	0.3059	0.0873	0.2073	12.27	11
Fe EDTA	10,12	0.5845	0.2311	0.3796	6.40	11

pH of this sample drifted above 10 during the addition of sodium hydroxide and the analysis could not be repeated. The results of Collaborator 13 for Samples 8 and 11 were rejected on the basis of the Dixon test (3). Results obtained for matched pairs of these 3 samples were necessarily excluded from pair statistical calculations. Average coefficients of variation were iron EDTA 3.52%, iron HEDTA 4.16%, iron DTPA 3.55%, iron EDDHA 3.52%, iron citrate 2.86%, and iron DPS 8.47%.

Pair statistics for the 6 iron chelates are listed in Table 3. All samples exhibit values for S_b which are considerably larger than S_r. This characteristic is reflected in the *F*-ratios which are all significant at the 95% confidence limit. Despite the apparent bias, the coefficients of variation are satisfactory for all except Samples 1 and 4. The relatively high standard deviations for Samples 1 and 4 may indicate that Fe DPS is quite sensitive to some of the techniques involved in the procedure.

Most collaborators' comments suggested the procedure was relatively straightforward and few problems were encountered. Several of the laboratories noted a difficulty in obtaining a stable pH of 8.5 when adding sodium hydroxide and were uncertain what pH range would be satisfactory. The wording of the method was changed after the collaborative study was completed to indicate an upper pH value which would be satisfactory. Although the method was designed to provide a final solution for AAS analysis which contained about 10 ppm iron, some of the collaborators made further dilutions of 2–3 fold because their instruments exhibited unacceptable sensitivity or linearity in the higher concentration range. This change did not cause significant differences.

Recommendation

It is recommended that the AAS method for chelated iron be adopted as official first action for EDTA, HEDTA, DTPA, DPS, EDDHA, and citrate chelates of iron. Because of the relatively large standard deviation for Samples 1 and 4, the procedure for iron DPS should be studied further.

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INDUSTRIAL CHEMICALS

Evaluation of Potential Analytical Approach for Determination of Polychlorinated Biphenyls in Serum: Interlaboratory Study

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Forty-four laboratories participated in evaluation of a method for determining polychlorinated biphenyls (PCBs) as AR 1254 in serum at the parts per billion level. The method involves deproteinating serum with methanol, extracting with hexane-ethyl ether, and eluting PCBs from deactivated silica gel for gas-liquid chromatographic determination with electron capture detection. Compounds are quantitated by using the Webb-McCall factors. Five serum pools, 4 containing in vivo-fortified PCBs (as AR 1254) or 8 in vitro-fortified chlorinated hydrocarbons (CHs), or both, were used. For PCB fortification levels of 9.89 (EP 2), 24.74 (EP 3), and 74.20 ppb (EP 4), interlaboratory coefficients of variation (CVs) for collaborators that adhered to protocol were 92.7, 67.6, and 25.8%, respectively. CVs on the same pools analyzed by the Centers for Disease Control (CDC) were 7.4, 7.8, and 4.6%, respectively. Average interlaboratory recoveries for pools EP 2, EP 3, and EP 4 were 138.1, 111.2, and 91.1%, respectively, and 99.8, 89.6, and 90.4%, respectively, for CDC on the same pools. There was a general decrease in the mean error for those laboratories that had participated in an earlier study in which they were allowed to use their own methods.

Earlier data, resulting from an interlaboratory study of bovine serum pools containing in vivo-bound polychlorinated biphenyls (PCBs) (as AR 1254) and in vitro-spiked chlorinated hydrocarbons (trial environmental pools) by the Centers for Disease Control (CDC) and 25 participating laboratories, indicated a wide discrepancy in participants' ability to obtain accurate and precise data for PCBs when using their own analytical methods (1).

A follow-up study was proposed in which participants would again be supplied with bovine serum pools containing in vivo-bound PCBs and in vitro-spiked chlorinated hydrocarbons (environmental pools). In addition to the serum pools, each participant would receive specific instructions for the analytical method to be used. The main objective was to see if discrepant values would thereby be reduced. Results of the study are reported here.

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Protocol for Interlaboratory Study

Preparation of Bovine Serum Pools

The methods used to prepare the serum pools were similar to those previously reported (2). The source of the in vivo-bound PCBs (as AR 1254) was a cow that had received oral doses of AR 1254 for 15 days, was allowed to approach equilibrium of storage without further dosing, and was then bled (3). The PCB level of stock serum was determined by using the analytical procedure presented in this paper ($N = 10$, $\bar{x} =$ 1441 ppb as AR 1254; SD, ± 26 ppb; CV, 1.8%). The levels of PCBs in the pools were targeted at a low, medium, and high range of analyte. The chlorinated hydrocarbons (CHs) chosen for in vitro fortification were selected on the basis of information obtained from the Environmental Protection Agency (EPA) Health and Nutrition Examination Survey II (HANES II) on pesticide levels in human serum (Sandra Strassman-Sundy, EPA, January 1981). CHs occurring in serum with a frequency of less than 1% were not included. Spiking levels were either the arithmetic mean reported by EPA (*p,p'*-DDT and *p,p'*-DDE) or levels comparable to spiking levels reported in a previous paper (2) (hexachlorobenzene, β-hexachlorocyclohexane, oxychlordane, heptachlor epoxide, *trans*-nonachlor, and dieldrin). CHs (purity 98–100%) were obtained from the EPA Pesticide Repository, Research Triangle Park, NC. A spiking solution containing the previously mentioned 8 CHs was prepared in acetone.

The environmental pools (EP_s) were prepared as follows:

EP 4: To a pool of essentially CH- and PCB-free bovine serum, an amount of serum from the cow dosed with AR 1254 was added to yield a

fortification level of approximately 75 ppb (ng/mL) PCBs. This pool was allowed to mix 72 h at 0°C.

EP 3: An aliquot of EP 4 was removed and added to a pool of essentially CH- and PCB-free bovine serum to yield a fortification level of approximately 25 ppb (ng/ml) PCBs. This pool was allowed to mix for 72 h at 0°C.

EP 2: An aliquot of EP 3 was removed and added to a pool of essentially CH- and PCB-free bovine serum to yield a fortification level of approximately 10 ppb PCBs. This pool was allowed to mix for 72 h at 0°C.

The 8-component CH spiking solution (50 mL acetone) was added to each of the above pools and allowed to mix for an additional 72 h at 0°C. The spiking solution constituted 1% of the final volume for EPs 4 and 2, and 0.95% of the final volume for EP 3.

EP 1: To a pool of essentially CH- and PCB-free bovine serum, acetone (50 mL) was added (1% of the final volume) and allowed to mix for 72 h at 0°C.

One additional pool, chlorinated hydrocarbons spike (CHS), was prepared by adding to essentially CH- and PCB-free bovine serum, 10 mL of the 8-component CH spiking solution (1% of the final volume) and allowing the solution to mix for 72 h at 0°C.

All pools were sterilized by using a Millipore filtration system, dispensed into 3 dram vials in a sterile environment, and stored at −30°C. Analyte fortification levels for each pool are shown in Table 1.

Table 1. Fortification levels (ppb) of environmental pools (EPs)

Analyte	EP 1	EP 2	EP 3	EP 4	CHS
PCBs (as AR 1254)[a]	—	9.89	24.74	74.20	—
Spike component[b]					
HCB	—	5.10	4.90	5.10	5.10
β-HCCH	—	6.09	5.86	6.09	6.09
Oxychlordane	—	3.12	3.01	3.12	3.12
Heptachlor epoxide	—	2.09	2.01	2.09	2.09
trans-Nonachlor	—	2.90	2.79	2.90	2.90
p,p'-DDE	—	18.04	17.35	18.04	18.04
Dieldrin	—	9.86	9.48	9.86	9.86
p,p'-DDT	—	1.90	1.83	1.90	1.90

[a] Fortification level defined as the projected concentration resulting from dilution with PCB-free serum of serum taken from a cow exposed to Aroclor 1254, i.e., a cow was exposed to AR 1254 and then bled, the PCB level in serum was determined and the serum was diluted.

[b] Fortification resulting from adding 8-component spiking solution in acetone.

Homogeneity Evaluation of Serum Pools

In our first study (2), we relied on long-term mixing to achieve homogeneity. In this study, we were interested in evaluating the homogeneity by analyzing sera that were dispensed at various times (4). Therefore, we performed duplicate analyses on the serum in the 1st, 100th, 200th, 300th, 400th, and 500th vial dispensed for each of the EPs.

Chromatography

Phases of the analytical procedures have been reported previously (2, 3, 5). Briefly, 4 mL serum is deproteinated with methanol and extracted with hexane–ethyl ether. Three fractions, 7 mL hexane; 15 mL hexane; and 15 mL benzene, are sufficient to elute CHs and PCBs from a 9.0 mm id column containing 3.0 g 3% deactivated silica gel. Column eluates were analyzed by gas-liquid chromatography, with a ^{3}H(Sc) or constant current ^{63}Ni electron capture detector interfaced to gas chromatographic columns of OV-17/QF-1 (1.5%/1.95%) and 3% SE-30, respectively. Peaks were quantitated manually by peak height (^{3}H) or electronically by peak area (^{63}Ni). Webb–McCall factors (6) were used in all PCB quantitations.

Results and Discussion

The results for PCBs (Table 2) were analyzed by 2-way analysis of variance to detect significant differences between vials (columns) and within vials (rows) (7). No significant difference was observed for PCBs between or within vials at the 5% level of significance.

The homogeneity of CHs in the pools was evaluated in a similar manner (Table 3). Although the EPs were in vitro-fortified with 8 CHs, only *p,p'*-DDE was evaluated for homogeneity because it was the only CH that could be confirmed as present in EP 1. It was also assumed that if *p,p'*-DDE demonstrated homogeneity, the remaining CHs would also be homogeneous. EP 1 was the only pool demonstrating nonhomogeneity among vials, on the basis of the statistical test performed. We believe, however, that the difficulty associated with determining *p,p'*-DDE at the concentration level found in EP 1 contributed significantly to the *F*-test failure. The EPs demonstrated sufficient homogeneity to be used in this study.

Characterization of Serum Pools by CDC

Characterized values for the EPs (PCBs) were established through a series of 20 analytical runs. In 5 of the 20 runs, characterized values for the EPs (CHs) were also established. A run consisted

Table 2. Homogeneity evaluation of environmental pools (PCBs, ppb)

Pool (EP)	Repetition	Vial No., order of dispensing 1	100	200	300	400	500
1	1	1.44	1.73	1.81	1.96	1.74	1.84
	2	1.99	1.74	1.82	1.95	1.80	1.65
Overall mean		1.78			F(column) = 0.49 (5.05)[a]		
SD		0.178			F(row) = 0.36 (6.61)		
CV, %		9.98					
2	1	10.16	10.46	10.46	10.75	10.82	9.86
	2	10.18	10.58	10.49	9.94	11.14	10.52
Overall mean		10.44			F(column) = 1.48 (5.05)		
SD		0.346			F(row) = 0.05 (6.61)		
CV, %		3.31					
3	1	24.40	24.16	24.34	24.22	LOST	23.34
	2	24.20	24.62	24.51	23.78		23.72
Overall mean		24.12			F(column) = 3.62 (6.39)		
SD		0.278			F(row) = 0.00 (7.71)		
CV, %		1.15					
4	1	62.54	62.92	63.76	64.58	62.26	62.58
	2	62.60	64.20	62.34	64.39	63.78	62.73
Overall mean		63.22			F(column) = 2.35 (5.05)		
SD		0.656			F(row) = 2.00 (6.61)		
CV, %		1.04					

[a] Critical value for F in parentheses.

of analyzing duplicate samples from 1 vial of EPs 1, 2, 3, and 4 and a single sample from 1 vial of CHS.

Under the analytical conditions used, CHs elute in all 3 fractions whereas PCBs elute only in fraction II (Figure 1 (standard) and Figures 2 and 3). Hexachlorobenzene, *trans*-nonachlor (trace), and *p,p'*-DDE elute with the PCBs (Figure 3). Elution of *p,p'*-DDE in more than 1 fraction is not uncommon (2). PCBs elute in fraction II only, as evidenced by comparing gas chromatographic traces of fractions I-III of EP 4 with CHs

Table 3. Homogeneity evaluation of environmental pools (CHs, ppb)[a]

Pool (EP)	Repetition	Vial No., order of dispensing 1	100	200	300	400	500
1	1	0.26	0.21	0.25	0.32	0.21	0.20
	2	0.26	0.21	0.24	0.28	0.20	0.19
Overall mean		0.24			F(column) = 38.07[b] (5.05)[c]		
SD		0.009			F(row) = 6.20 (6.61)		
CV, %		3.90					
2	1	16.41	16.58	16.45	17.66	18.00	14.39
	2	16.42	16.48	16.16	15.36	18.26	14.78
Overall mean		16.41			F(column) = 4.86 (5.50)		
SD		0.721			F(row) = 0.38 (6.61)		
CV, %		4.40					
3	1	12.56	16.22	15.23	15.16	LOST	15.04
	2	16.47	15.23	15.95	15.83		15.10
Overall mean		15.28			F(column) = 0.25 (6.39)		
SD		1.384			F(row) = 0.50 (7.71)		
CV, %		9.06					
4	1	13.06	14.47	13.79	14.35	14.50	14.04
	2	14.23	13.18	13.85	15.81	14.74	12.61
Overall mean		14.05			F(column) = 1.21 (5.05)		
SD		0.848			F(row) = 0.04 (6.61)		
CV, %		6.04					

[a] Based on *p,p'*-DDE.
[b] Significant at $P \leq 0.05$.
[c] Critical value for F in parentheses.

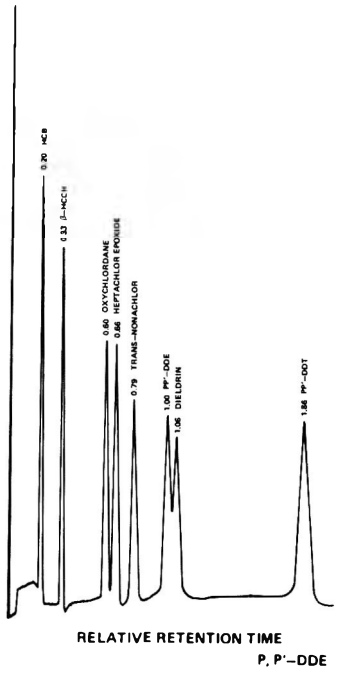

Figure 1. Chromatogram of 8-component chlorinated hydrocarbon standard: HCB (27.6 pg), β-HCCH (105.8 pg), oxychlordane (55.2 pg), heptachlor epoxide (55.2 pg), *trans*-nonachlor (57.5 pg), *p,p'*-DDE (52.9 pg), dieldrin (50.6 pg), *p,p'*-DDT (151.8 pg). GC conditions: column temperature 200°C; nitrogen flow rate 50 mL/min; 6 ft × 4 mm id glass column packed with OV-17/QF-1 (1.5%/1.95%) on 80-100 mesh Chromosorb W HP.

(Figures 2-4). We continue to observe extraneous peaks in those fractions from silica gel that contain most of the CHs; the interferences are more extensive than previously experienced (2). Because of interferants in fraction III, β-hexachlorocyclohexane was confirmed after an additional elution from a micro-Florisil column with 6% ethyl ether-petroleum ether (8) and electron capture analysis by using a SE-30 column (see inset Figures 2 and 3). Recovery of the in vitro-fortified CHs is shown in Table 4 for each pool. Present recoveries are as good or better than those observed in an earlier study (2) except for *trans*-nonachlor and *p,p'*-DDT. *p,p'*-DDT concentration in this study is about one-fourth the level of the earlier one. We believe that preparing the spiking solution directly in acetone and using a keeper solution (1% paraffin oil in hexane) (9) for all solvent evaporation steps enhanced the recovery in this study, particularly of hexachlorobenzene.

Our characterized values for the PCBs (N = 40 for pools EP 1-EP 4) were evaluated by using one-way analysis of variance (Table 5). In the one-way format, the run numbers (1-20) were designated as the classification variable in order to estimate within-run and among-run components of variance and total variance (sum of estimates of within and among components). These values are without the contribution of peak 98 (6), which was not considered because the adsorption column used elutes >90% of the extracted *p,p'*-DDE (RRT = 100) in this fraction and the gas chromatographic column will not resolve these 2 peaks under the analytical conditions used. Although the loss of peak 98 is not desirable, in our analysis of environmental samples and the analysis of human samples with known PCB exposure (10), peak 98 has not been found to contribute significantly to PCB levels. On the basis of fortification levels of EPs 2, 3, and 4, the average recoveries were 99.8, 89.6, and 90.4%, respectively, although the contribution of peak 98 was not considered. Our characterized value for CHS ($\bar{x} \pm$ SD) as Aroclor 1254 was 1.65 ± 0.176 ppb without the *p,p'*-DDE (peak 100). This value is not too different from the characterized value of EP 1 (Table 5), which is as it should be if the adsorption column is performing properly. Overall, the coefficients of variation (CVs) for CDC were noticeably lower in this study than in the earlier one (2), and the studies differ only with regard to the adsorption chromatography.

Characterization of EPs by Collaborators

We solicited the participation of laboratories from federal (F), state (S), and private (P) sectors. Each collaborator was supplied with an analytical kit that contained the following: (1) 2 vials randomly selected (~9 mL each) from each of serum pools EPs 1-4; (2) 1 vial (~4 mL) of CHS serum pool; (3) 1 vial of neat Aroclor (AR) 1254, Lot AK-38; (4) 50 g Woelm silica gel (70-150 mesh), Lot 2417; (5) a copy of CDC Laboratory Update 81-108 (11) that detailed the method; (6) a representative gas chromatographic trace of AR 1254 illustrating resolution of the standard obtained under CDC operating conditions.

Collaborators were asked to analyze 1 vial of EPs in duplicate and 1 vial of CHS pool for PCBs only, using appropriate standards prepared from the Aroclor reference material supplied and the method detailed in CDC Laboratory Update 81-108. Deviations from the prescribed method and gas chromatographic traces representative of each pool were to be reported by each collaborator when analytical results were returned.

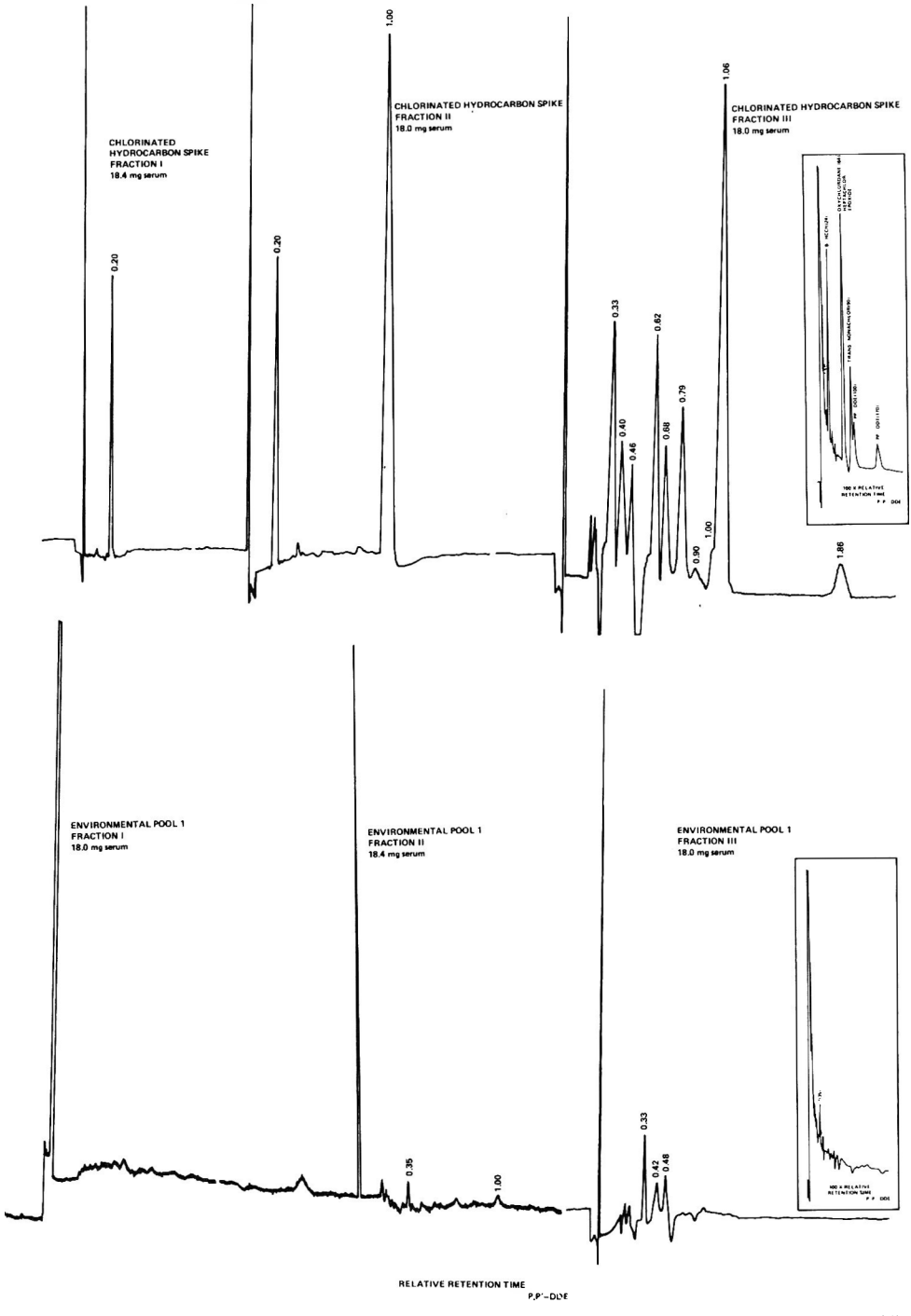

Figure 2. Chromatograms of CHS fractions I-III and EP 1 fractions I-III. See Figure 1 for peak identification and GC conditions.

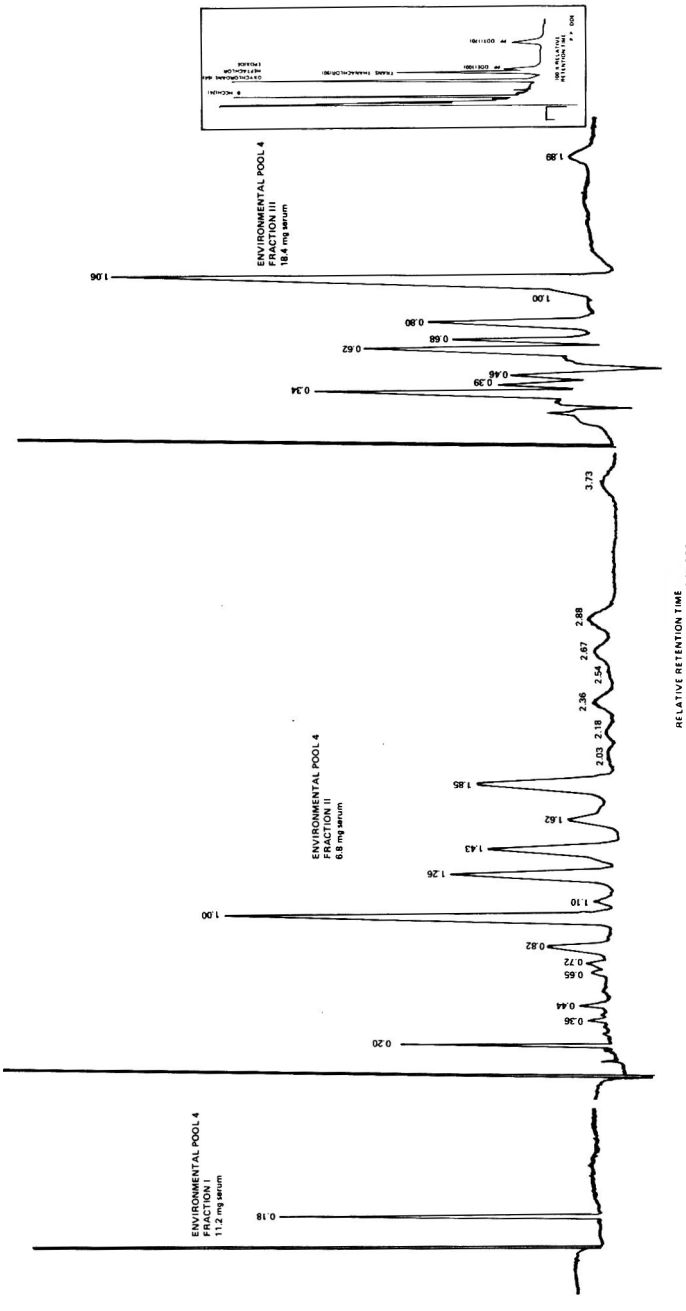

Figure 3. Chromatograms of EP 4 fractions I–III. See Figure 1 for peak identification and GC conditions.

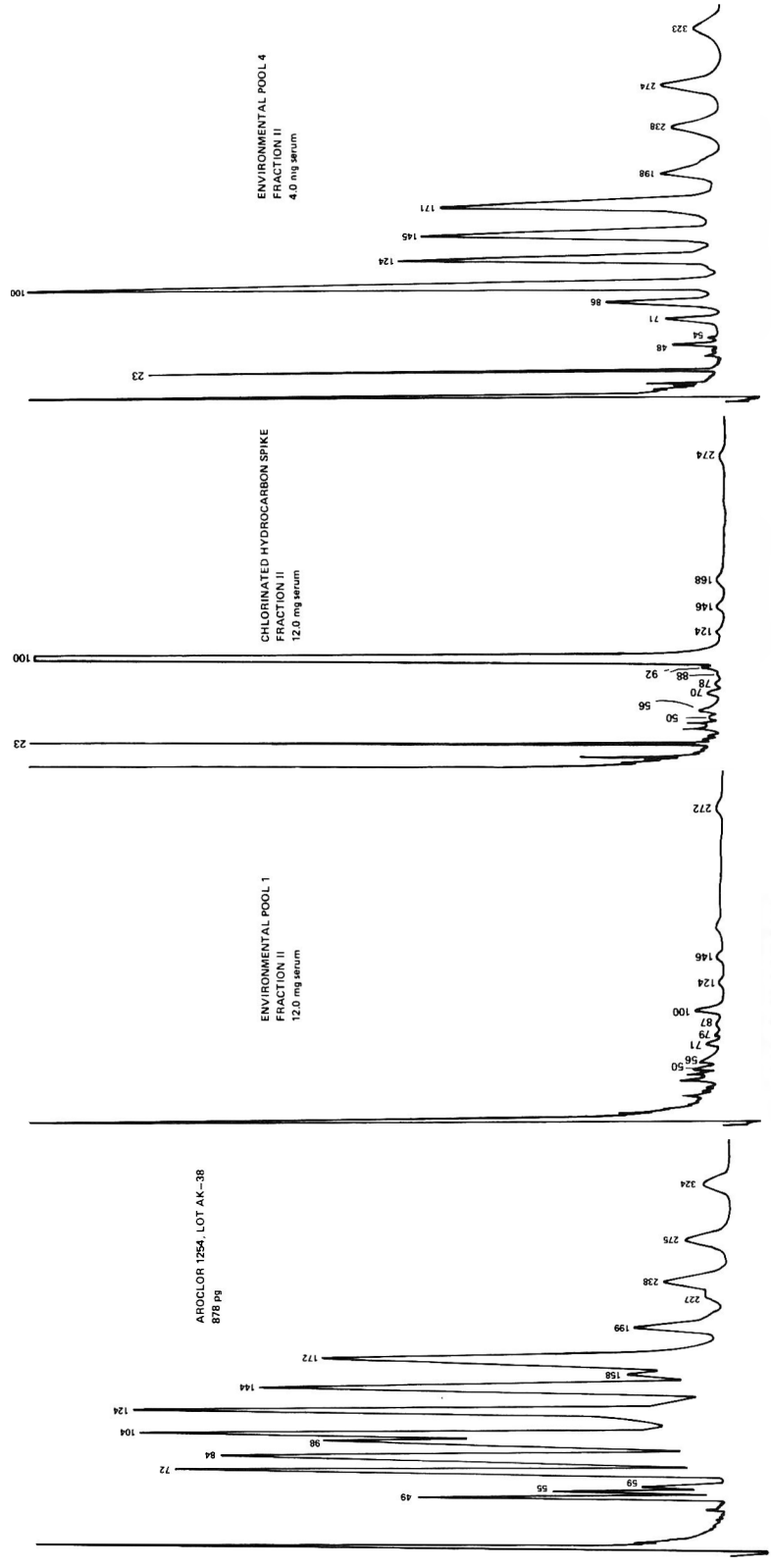

Figure 4. Chromatograms of Aroclor 1254 standard and fraction II for EP 1, CHS, and EP 4. GC conditions: column temperature 195 °C; nitrogen flow rate 20 mL/min; 6 ft × 2 mm id glass column packed with 3% SE-30 on 80–100 mesh Gas-Chrom Q.

Table 4. Recovery (%) of in vitro-fortified chlorinated hydrocarbons in environmental pools [a]

Analyte	EP 2	EP 3	EP 4
HCB	82.8 ± 3.8	77.1 ± 2.0	65.0 ± 6.4
β-HCCH	60.4 ± 9.2	69.6 ± 6.0	64.9 ± 4.2
Oxychlordane	71.0 ± 10.0	84.6 ± 5.2	77.1 ± 3.7
Heptachlor epoxide	71.0 ± 11.5	85.2 ± 5.8	77.6 ± 4.6
trans-Nonachlor	69.3 ± 11.4	84.8 ± 9.0	81.4 ± 7.8
p,p'-DDE	91.0 ± 6.6	88.0 ± 6.2	77.8 ± 4.7
Dieldrin	88.0 ± 11.4	95.4 ± 8.9	76.9 ± 6.3
p,p'-DDT	70.1 ± 9.7	73.0 ± 5.2	73.0 ± 8.7

[a] Mean percent ± SD; $N = 6$ analytical runs of duplicates for EPs 2 and 4, $N = 5$ analytical runs of duplicates for EP 3.

Eighty laboratories received the analytical kit but only 44 reported data.

Although collaborators had been supplied with copies of the method, they tended to vary the prescribed procedure. Two collaborators, P-3 and P-7, varied the serum volume. One collaborator (P-7) did not deproteinate the serum with methanol, and used isooctane for extraction.

Although the method prescribed the use of a mechanical rotator in the extraction step, S-3 and S-7 extracted by hand shaking. S-9 used a vortex mixer; S-13 combined hand shaking and sonication, and P-10 used sonication only.

Collaborators varied the activation temperature of silica gel (none to >150°C), and the activation time (none to >48 h). We have not observed an increase in water loss from silica gel heated beyond 24 h at 130°C. Silica gel has been reported, however, to lose water continuously when heated from 100 to 1000°C (12).

Adsorption columns used varied from 7 to 10 mm id, except that 2 collaborators, S-7 and P-10, used columns of 11 and 16 mm id, respectively. Significant variations in adsorption column from 9 mm id would affect the volume required to quantitatively elute PCBs.

Only one collaborator (P-7) used an amount of silica gel (0.9 g) that deviated from the method.

Two collaborators (S-21, P-7) reported PCB elution volumes of 30 mL, which exceeds what we have found necessary to quantitatively elute PCBs from the adsorption column specified.

All collaborators used electron capture detectors for their determinations. ^{63}Ni and ^{3}H sources were used 97.6 and 2.4% of the time, respectively. One collaborator (S-18) did not adhere to protocol in that temperature programming was used instead of isothermal conditions.

Variations that caused us not to consider data from certain laboratories for statistical analysis were as follows: (*1*) failure to use liquid phases of the methyl silicone type (6) (S-1, S-19, and P-9); (*2*) use of a capillary column instead of a packed column (S-2); (*3*) failure to use Webb–McCall factors in quantitation (6) (S-1, S-12, S-13, S-15, P-3, P-5, P-7, P-10, and P-11); (*4*) failure to analyze duplicate aliquots from each vial (S-21, P-12); (*5*) failure to provide sufficient information to ascertain to what extent the method was followed (S-10, P-1, and P-6).

Table 6 contains the data submitted by all collaborators regardless of whether they adhered to protocol. Several collaborators had participated in the initial study (1).

Our primary objective was to obtain data that would facilitate evaluation of the proposed procedure. We examined statistically only the data submitted by laboratories that adhered to protocol, particularly with regard to the type of gas chromatographic liquid phase and quantitation by using Webb-McCall factors. In all statistical evaluations, values reported as zero or less than 1 are considered as 1.0. Statistical treatment of data submitted by collaborators

Table 5. Statistical analysis of CDC-characterized values obtained for PCBs (ppb) in environmental pools

Pool	FL [a] (ppb)	*N* (runs)	Range	Mean	CV (%) for within-run component of variance	CV (%) for among-runs component of variance	CV (%) for within- and among-runs component of variance
EP 1	—	20	1.26–2.49	1.60	18.8	3.6	19.2
EP 2	9.89	20	8.05–11.36	9.87	4.8	5.7	7.4
EP 3	24.74	20	18.70–25.56	22.16	3.4	7.0	7.8
EP 4	74.20	20	60.06–72.40	67.14	2.6	3.8	4.6

[a] FL = fortification level defined as the projected concentration resulting from dilution with PCB-free serum of serum taken from a cow exposed to Aroclor 1254, i.e., a cow was exposed to AR 1254, and then bled, the PCB level in serum was determined and the serum was diluted.

Table 6. Collaborator results for total PCBs (ppb) in environmental pools (EPs) and chlorinated hydrocarbon spike (CHS)

	EP 1		EP 2		EP 3		EP 4		
Lab. code	Orig.	Dup	Orig.	Dup	Orig.	Dup	Orig.	Dup	CHs
S-1[a]	1.6	1.7	7.2	5.9	13.04	12.93	43.7	41.7	1.9
S-2[a]	ND[b]	ND	10.7	13.4	16.0	18.0	61.7	54.8	<1
S-3 (S-14)[c]	7.4	6.5	17.7	16.4	28.2	28.2	60.7	62.8	6.4
S-4 (S-5)	1.2	1.0	8.5	7.5	27	25	62	65	0.3
S-5 (S-19)	2.82	4.11	10.0	11.8	23.1	24.6	62.5	63.2	1.7
S-6	47.14[d]	61.47[d]	69.27	76.78	91.46	121.14	128.73	122.87	52.67
S-7 (S-2)	2.74	3.13	11.59	9.76	35.09	31.85	81.82	73.14	1.89
S-8	8.2	8.4	11.5	10.8	54.8	55.6	88.4	86.0	4.8
S-9 (S-8)	3.14	3.51	16.46	14.25	33.97	29.54	82.45	99.98	0.93
S-10[a]	27	63	148	127	206	302	690	628	89
S-11	3.1	2.3	11.9	13.6	36.8	27.2	79.0	77.6	15
S-12[a]	2.5	2.3	11.2	11.6	19.6	27.7	87.9	81.9	12.7
S-13[a]	NDR[e]		17.05	17.12	NDR		NDR		NDR
S-14 (S-12)	2.1	2.5	9.3	9.6	22.0	20.9	65.3	65.8	2.5
S-15[a]	—[f]	—[f]	20.91	23.63	39.20	39.36	164.60	136.72	—[f]
S-16 (S-17)	1.4	1.9	8.6	7.3	16.0	19.7	49.3	56.0	1.2
S-17 (S-7)	1.29	2.06	8.68	9.65	19.84	25.74	54.58	56.95	115.95[d]
S-18	<1	<1	8.1	8.1	17.1	14.5	53.4	63.4	2.3
S-19[a]	8.2	5.2	8.3	7.1	15.0	12.0	36.0	49.0	17.0
S-20	47.4[d]	42.8[d]	137[d]	141[d]	236[d]	219[d]	387[d]	445[d]	66
S-21[a]	2.7	2.6	10	NA[g]	23	NA	77	NA	NA
S-22 (S-18)	2.1	2.3	11.4	11.8	25.2	27.8	68.1	70.5	lost
S-23 (S-16)	3.40	3.14	11.58	11.34	21.34	24.65	58.06	56.60	2.0
S-24	<1.50	<1.20	11.1	12.0	24.0	24.3	54.4	71.8	<1.8
P-1[a]	3.0	<3.0	8.0	8.0	19.0	22.0	58.0	53.0	3.0
P-2	1.15	0.93	7.19	6.26	11.5	10.4	36.6	40.8	3.5
P-3[a] (P-5)	<5	<5	10	18	20	36	64	71	<5
P-4	3.48	4.60	10.4	10.1	24.6	19.9	66.7	66.5	22.5
P-5[a]	15.7	6.3	13.3	7.0	31.7	32.1	86.1	101	3.8
P-6[a]	0.02	0.02	0.04	0.03	0.06	0.06	0.16	0.15	0.02
P-7[a] (P-1)	0	0	6	8	26	21	84	78	0
P-8 (P-3)	<1	<1	8.4	9.0	19.2	16.8	52.5	57.2	<1
P-9[a]	1	4	13	12	17	20	43	41	7
P-10[a]	151	158	150	118	177	125	238	184	38
P-11[a]	1.9	2.1	19	17	48	33	140	150	1.7
P-12[a]	0	NA	13.2	NA	18.4	NA	45.0	NA	0
P-13	0.47	3.1	21.4	19.3	19.4	18.4	60.8	82.4	60.8
P-14	1.20	0.66	10.4	9.7	18.4	19.9	65.1	64.9	0.92
P-15	2.7	2.6	12.5	16.3	28.0	26.3	80.0	87.1	13.7
P-16[a]	ND	ND	15.12	19.90	42.8	lost	105.95	113.10	ND
F-1	0.36	0.78	9.1	9.3	24	18	63	58	0.26
F-2 (S-15)	0.62	0.32	11	6.5	24	15	58	81	0.77
F-3	4	2	13	13	18	16	43	50	<2
F-4	3.20	3.87	11.03	12.78	25.57	25.04	65.93	60.56	3.73
N	48		50		50		50		24
Mean	2.54		13.66		27.50		67.60		11.12
Mean % rec.	—		138.1		111.2		91.1		—
SD	1.926		12.665		18.577		17.510		19.668
CV, %	75.9		92.7		67.6		25.8		176.8
Fortification level (PCBs as AR 1254)	—		9.89		24.74		74.20		—
CDC-characterized values									
N	40		40		40		40		20
Mean	1.60		9.87		22.16		67.14		1.65
Mean % rec.	—		99.8		89.6		90.4		—
SD	0.306		0.736		1.738		3.114		0.176
CV, %	19.1		7.4		7.8		4.6		10.6

[a] Laboratory did not follow protocol.
[b] ND = not detected at collaborators' lower detection limits.
[c] Laboratory code when it participated in initial study (1) (in parentheses).
[d] Outlier as described by Youden and Steiner (13).
[e] NDR = no data reported.
[f] No data reported due to extreme interferences.
[g] NA = not analyzed.

Table 7. Total error (%) values for environmental pools (PCBs, ppb)

Results	Pool	N[a]	True value[b]	Mean	Absolute value mean error	Std dev.	Total error (%)[c]
Collaborator values	EP 2	50	9.89	13.66	3.77	12.665	294
	EP 3	50	24.74	27.50	2.76	18.577	150
	EP 4	50	74.20	67.60	6.60	17.510	56
CDC values	EP 2	40	9.89	9.87	0.02	0.736	15.0
	EP 3	40	24.74	22.16	2.68	1.738	24.8
	EP 4	40	74.20	67.14	7.06	3.114	17.9

[a] Only those collaborators that adhered to protocol are included, i.e., 25 collaborators performed duplicate analyses on each pool, using prescribed method. CDC performed 20 analytical runs of duplicates for each pool, using prescribed method.

[b] For true value substituted fortification level defined as the projected concentration resulting from the dilution with PCB-free serum of serum taken from a cow exposed to Aroclor 1254, i.e., a cow was exposed to AR 1254 and then bled, the PCB level in serum was determined and the serum was diluted.

[c] Total error (%) = 100 × (absolute value of mean error + 2 std dev.)/true value.

adhering to protocol is also shown in Table 6.

The interlaboratory average recovery values for EP 2 (9.89 ppb), EP 3 (24.74 ppb), and EP 4 (74.20 ppb) were 138.1, 111.2, and 91.1%, respectively. With the exception of EP 4, recoveries obtained by participants are higher than those obtained by CDC. The corresponding interlaboratory CV values of 92.7 (EP 2), 67.6 (EP 3), and 25.8% (EP 4) are higher than those obtained by CDC and indicate a lack of precision among laboratories especially for EP 2 and EP 3.

The interlaboratory CVs obtained for EP 1 and CHS, 75.9 and 176.8%, respectively, were higher than CDC values as were the interlaboratory mean values for PCBs in those pools, 2.54 and 11.12 ppb, respectively. The CHS pool mean of 11.12 ppb for participants was almost 7 times higher than the CDC value of 1.65 ppb. This is somewhat surprising because CHS and EP 1 are composed of serum from the same source. We suspect that some participants inadvertently included *p,p'*-DDE in their PCB quantitation, although instructed not to do so, or may have eluted CHs other than *p,p'*-DDE in the PCB fraction; other participants may have experienced unusually high reagent blank interferences. All of these factors could account for the high values reported for CHS.

We detected trace amounts of *p,p'*-DDE and PCBs in EP 1. In an earlier study, the presence of PCBs was confirmed by mass spectrometry in serum similar to EP 1 (1).

There are no rigid guidelines on what constitutes acceptable interlaboratory precision for analyses of this type. However, an average interlaboratory CV of about 15% has been reported for 12–22 laboratories analyzing 3–6 organic chlorine pesticides in blood (14). The type of analysis under consideration here, while similar in many respects, presents additional analytical problems to those encountered when determining CHs only.

A criterion for judging the acceptability of methods has been proposed (15) in which the percent of total error (TE%) is used. A ranking is established, e.g., those methods whose total errors are 25% or less are considered excellent, those whose errors are 50% or less, acceptable, and greater than 50%, unacceptable. These limits are arbitrary and tend to vary, depending on the analyte, concentration, and matrix. Total error values for collaborators have been computed and are shown in Table 7. These values are unusually high except for EP 4; however, all values would be unacceptable according to McFarren's criteria. Although McFarren et al. (15) do not apply their evaluation technique to data generated by single laboratories, some investigators have done so (16, 17). We have also included in Table 7 data generated by CDC and evaluated for TE%. Although McFarren et al. propose no criteria for data generated within a single laboratory, one would expect the limits to be narrower. Our TE% values for EPs 2, 3, and 4 are 15.0, 24.8, and 17.9, respectively.

One of our objectives was to determine if there were fewer discrepant values when collaborators were supplied with a method as opposed to using their own. In Figure 5, we compare the mean error of values for collaborators that participated in both studies. In general, the mean error is greater for the first study (TEPs 2 and 3) than for the second (EPs 2, 3, and 4). This is confirmed by comparing the algebraic sum of the mean errors for TEP 2 (+101.62) and TEP 3 (+152.0) with

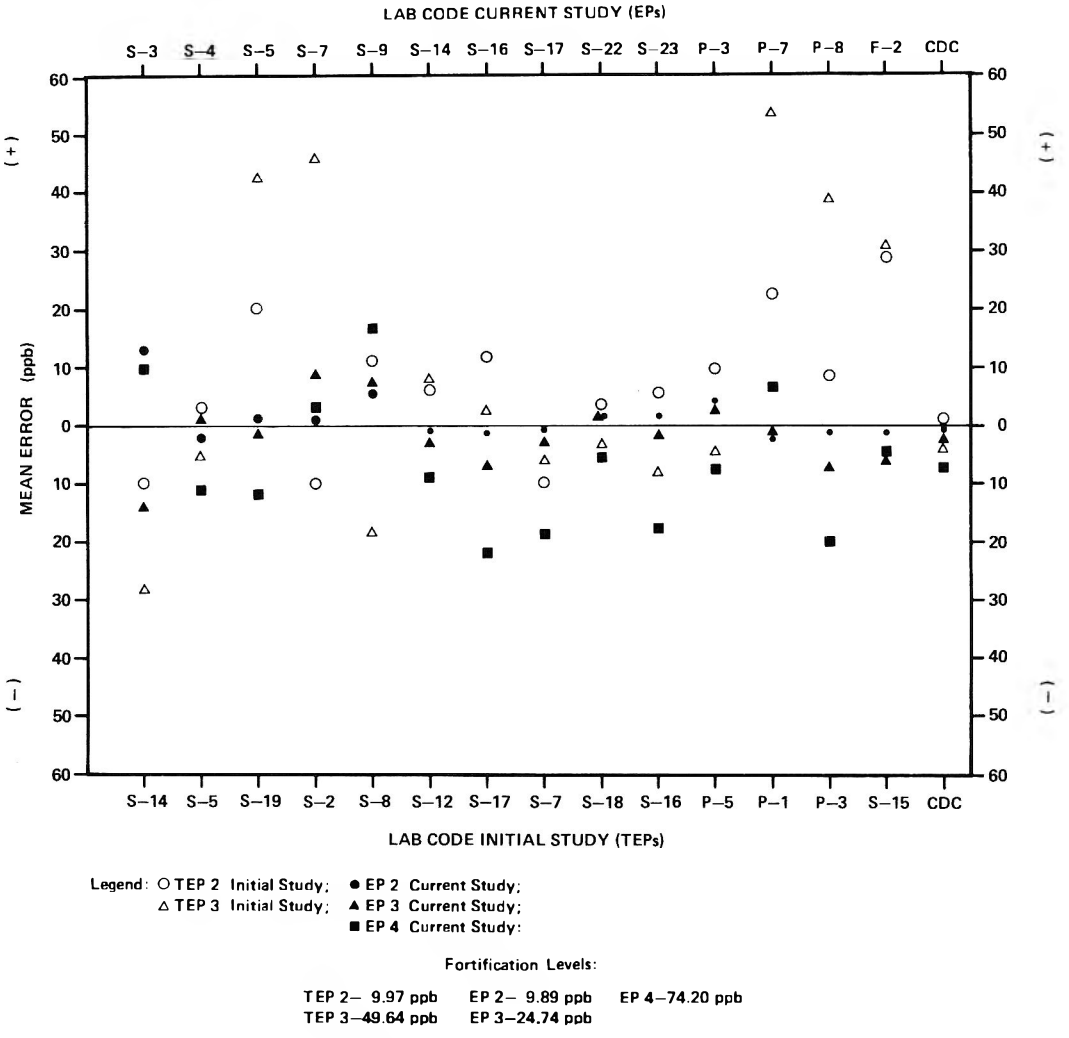

Figure 5. Mean error (algebraic difference between lab. mean of pool and fortification level of pool) values for collaborators that participated in both studies.

the sums for EP 2 (+17.72), EP 3 (+7.65), and EP 4 (−86.39). Comparable values for CDC were (+1.41), (−3.63), (−0.02), (−2.58), and (−7.06), respectively.

The overall performance of the method for determining PCBs in the presence of CHs is not good using TE% and the criteria proposed. There is evidence, however, of an overall improvement in accuracy reflected in the decrease of mean error for those laboratories that have participated in both studies (1). Laboratories, as a whole, performed better using the method under investigation than they did using their own methods. Our investigation of the analytical methodology in this area is scheduled to follow a 3-step approach. First, participants, using their own methods, would analyze a set of bovine serum pools containing in vivo-bound PCBs (as AR 1254) and a select number of in vitro-spiked CHs (reported in ref. 1). Second, participants would again be asked to evaluate bovine serum pools containing in vivo-bound PCBs (as AR 1254) and a select number of in vitro-spiked CHs, using a proposed method, this study. Third, a repeat evaluation of the proposed method under the auspices of the AOAC is proposed.

Collaborator Comments and CDC Response

Collaborator S-6 reported difficulty in setting up a data system to integrate PCB peak 174. We also had difficulty with this peak because of its

broad tailing base. We have solved this problem by using the grouping function within the Varian CDS 111C program (3).

Collaborator S-7 reported that concentrating the hexane–ethyl ether extract by using a nitrogen stream results in moisture in the sample. S-7 laboratory personnel also believe that a humid laboratory environment may have affected PCB elution from silica gel. We have not observed moisture following solvent reduction of the organic extract. We suspect that, in removing the organic phase, they may have removed a portion of the interface which does contain water and methanol. While using the prescribed method, we have not experienced any untoward effects from environmental conditions, however, the procedure we used in the first study (2) showed this sensitivity.

S-9 laboratory personnel felt that the final volume of sample extract (0.5 mL) before adsorption chromatography was too small, making the transfer difficult. As a general rule, in our adsorption chromatography studies, we try to keep the sample volume <5% of the elution volume.

Collaborator S-15 reported problems with extraneous peaks in CHS and EP 1. Other collaborators also reported problems with these 2 pools. We acknowledge the presence of trace amounts of naturally occurring *p,p'*-DDE and PCBs in EP 1. The extraneous peaks that some collaborators observed may be attributed to reagents used in the procedure.

S-16 personnel indicated that SilicAR CC-4 is superior to silica gel in retaining CHs. They believe that serum of low lipid content does not need adsorption chromatography, because the CHs are not completely separated. In our hands, SilicAR CC-4 was comparable but not superior to silica gel. There is evidence of CH retention by silica gel when fractions II and III of CHS are compared in Figures 2 and 4. We have encountered problems with column and detector fouling when analyzing serum samples extracted by this method (11) without adsorption chromatography.

S-18 personnel expressed concern over the use of specific Webb-McCall factors with Aroclors that have lot numbers different from those on which the factors were originally determined. They also believe that exclusion of PCB peaks 98 and 104, because of the co-elution of *p,p'*-DDE, compromises the accuracy of the serum determination. Steichen et al. (18), using the *t*-test, have compared several available lots of Aroclor 1254 and have found them not to be significantly different from characterized Aroclor standards (6, 19) at the 80% confidence level. Steichen et al. (18) also report that AR 1254 characterized by Webb-McCall (6) was Lot AK 38.

The loss of peak 98 is not analytically desirable, but in incidents of known human exposure to PCBs, PCB peak 98 does not make a significant contribution to the total in vivo PCB residues (10). It has also been shown in Canadian aquatic birds (20) and sea birds from the Pacific Ocean (21) that the PCB contribution to *p,p'*-DDE is not significant. Peak 104 was not observed when serum of the dosed cow was analyzed by the method being evaluated.

P-5 personnel suggested that the method be evaluated using capillary systems. We readily admit that a glass capillary gas chromatographic system interfaced to a mass spectrometer is the analytical mode best suited for analyzing compounds of this type. We also believe, however, that a more routine method with greater accessibility is needed at this time.

Collaborator P-9 suggested the use of acetonitrile and Florisil instead of methanol and silica gel. We have not investigated the use of acetonitrile for denaturing the serum; some investigations, however, have reported using hexane-saturated acetonitrile for extracting blood (22). A participant (S-8) in our initial study (1) also used acetonitrile for extraction. We have encountered few samples of human or environmental origin that do not contain PCBs and CHs. Silica gel is superior to Florisil in separating PCBs from CHs.

P-11 personnel expressed concern that the response of their in-house AR 1254 standard was 1.6 times that of the AR 1254 supplied by CDC. Examination of their gas chromatographic traces with respect to PCB peak distribution revealed the following: Chromatograms from Collaborator P-11 show PCB peaks with relative retention times (RRT) to DDE × 100 of 66, 75, 82, 94, 100 . . . , whereas the CDC-supplied AR 1254 shows PCB peaks with RRT to *p,p'*-DDE × 100 of 46, 52, 54, 68, 80, 94, 100 . . . . The major differences are in those peaks with RRT <120. The CDC pattern is more similar to the Webb-McCall pattern (6). The difference between the 2 standards could be attributed to some chemical degradation in the standard of Collaborator P-11 or to a difference in lot.

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Rapid Screening Procedure for Pesticides and Polychlorinated Biphenyls in Fish: Collaborative Study

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A simplified rapid method is presented to determine organochlorine pesticides and polychlorinated biphenyls in fish. Samples are mixed with sodium sulfate and blended with petroleum ether. A portion of the blend is placed on a miniaturized Florisil column and compounds are eluted with mixtures containing 6 and 15% ethyl ether in petroleum ether. Gas-liquid chromatography with electron capture detection comparable to the official methods (29.018) is used for determination of residues. Each of 7 collaborators reported results in duplicate for 10 samples containing one or more of the following compounds: *p,p'*-DDD, *p,p'*-DDE, *p,p'*-DDT, dieldrin, heptachlor epoxide, Aroclor 1254, and Aroclor 1260. Recoveries ranged from 79.3 to 100.7%. The coefficients of variation between laboratories ranged from 6.3 to 20.6%. This method allows up to 80% reduction in reagents and up to 50% reduction in analytical time, and requires less laboratory space than current official methods. The method has been adopted official first action.

Analysis for organochlorine pesticides and polychlorinated biphenyls (PCBs) in fish is part of the monitoring program of the Food and Drug Administration (FDA). Current multiresidue methodology (1, 2) used to determine these compounds is often time-consuming and costly. This study reports a reliable rapid procedure to screen for organochlorine pesticide and PCB residues in fish.

Sample, sodium sulfate, and petroleum ether are blended and a sufficient volume of extract is obtained to determine percent fat in fish and to perform the analysis and additional determinations if needed. A miniaturized Florisil column previously tested by Erney (3) for sample extract cleanup is used in a fashion similar to the Florisil column described in the *Pesticide Analytical Manual* (PAM) (2). Gas-liquid chromatography (GLC) with electron capture detection, which is considered equivalent to PAM methodology, is used for residue determination.

The proposed procedure reduces reagent requirements by as much as 80% and analytical time by as much as 50%, compared with the PAM methodology.

This report of the Associate Referee was presented at the 96th Annual International Meeting of the AOAC, Oct. 25-28, 1982, at Washington, DC.

The recommendation of the Associate Referee was approved by the General Referee and Committee E and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

Received September 1, 1982.

Chlorinated Pesticides and Polychlorinated Biphenyls in Fish

Gas-Liquid Chromatographic Method

Official First Action

29.D01 ***Principle***

Chlorinated pesticides and polychlorinated biphenyls (PCBs) are extd from prepd fish sample with pet. ether, cleaned up on Florisil column, and detd by GLC against ref. stds.

See **29.002, 29.005, 29.008-29.010** for general app., reagents, and technics.

29.D02 ***Apparatus***

(a) *Gas chromatograph.*—With on-column injection system, 6 ft glass column (4 mm id), packed with 10% DC-200 on 80–100 mesh Chromosorb WHP, and electron capture detector. Other liq. phases such as 5% OV-101 on suitable supports may be substituted if known to give adequate resolution for compds present in samples.

Linearized ^{63}Ni detector capable of producing ½ scale deflection for 1 ng heptachlor epoxide is suggested; however, other equiv. electron capture detectors may be used. Operate GLC in accordance with manuf. directions, adjusting to provide necessary response and resolution.

(b) *Chromatographic tube.*—10 mm id × 300 mm column with Teflon stopcock, coarse fritted disk, ₹24/40 top joint (Kontes Glass Co. K-420550, or equiv.).

(c) *Kuderna-Danish concentrators.*—Snyder distg column (Kontes K-503000-0121); 125 mL K-D flask (Kontes K-570001-9010) (special item)

⅋ 19/22 lower joint; 10 mL concentrator tube (Kontes K-570050-1025).

(d) *Micro Snyder column.*—Kontes K-569251, ⅋ 19/22.

29.D03 *Reagents*

(a) *Florisil.*—PR grade, 60–80 mesh (Floridin Co.). Must meet **29.002(i)** specifications.

(b) *Solvents.*—Pet. ether, ethyl ether, hexane, and alcohol, known to be suitable for pesticide residue detn.

(c) *Glass wool (Pyrex).*—Must be free of interference with electron capture detection.

(d) *Sodium sulfate.*—Anhyd., granular, reagent grade, free of interference with electron capture detection.

29.D04 *Extraction*

Weigh 20 g thoroly ground and mixed sample into metal blender cup. Moisten 40 g granular Na_2SO_4 with pet. ether and add to sample. Mix sample, using stirring rod, let stand 20 min, and mix again. Add 100 mL pet. ether to sample and blend 1–2 min. (Lourdes blender in series with rheostat set at 40–60%, or equiv., may be used.) Centrf. balanced sample cup 1–2 min at ca 2000 rpm to obtain clear pet. ether ext. Place glass wool plug in funnel, overlay with 20 g granular Na_2SO_4, and place funnel in 250 mL vol. flask. Decant pet. ether ext thru Na_2SO_4 into vol. flask. Mix sample again with stirring rod, add 100 mL pet. ether, and ext as before. Repeat using 70 mL pet. ether. Dil. to vol. with pet. ether.

Transfer 25 mL aliquot to tared 100 mL flat bottom extn flask. Place flask on steam bath to evap. solv., leave addnl 30 min on steam bath, remove, and cool. Weigh flask and det. % fat in fish.

For fish contg <10% fat, transfer 25 mL aliquot to 125 mL K-D concentrator. For fish contg >10% fat, take aliquot contg not >200 mg fat. Add several granules of 20–30 mesh carborundum and conc. to ca 3 mL on steam bath. Let cool and remove Snyder column. Rinse concentrator with two 1 mL portions of pet. ether and, using only current of air, conc. sample to 3 mL for transfer to Florisil column.

29.D05 *Florisil Cleanup*

Use 4 g Florisil adjusted for lauric acid value (*JAOAC* **51,** 29(1968)). Add Florisil to 300 × 10 mm id chromatgc tube and add Na_2SO_4 to ht 2 cm above Florisil. Completely open stopcock, tap tube to settle adsorbent, and mark tube 1 cm above Na_2SO_4 layer.

Add 20–25 mL pet. ether wash to Florisil column; as solv. level reaches mark, place 125 mL K-D flask under column. Using disposable Pasteur pipet, transfer 3 mL sample to column, and wash tube with 1 mL pet. ether and add wash to column. Solv. level must not go below mark. Temporarily close stopcock if necessary. Add 35 mL pet. ether–ether mixt. (94 + 6) and elute PCBs and DDT, and its analogs. When solv. level reaches mark, change K-D flask, and add 35 mL pet. ether–ether (85 + 15) to elute compds such as dieldrin and endrin. Add several granules of carborundum to first concentrator, attach Snyder column, and carefully conc. on steam bath. Let concentrator cool, remove Snyder column and evap. solv. under air to appropriate vol. for GLC detn. Fractions contg mixt. of PCBs and chlorinated compounds such as DDE may require addnl sepn technics.

29.D06 *Additional Cleanup*

Often addnl cleanup is required for second fraction (85 + 15) to prevent deterioration of GLC column. Conc. pet. ether–ether (85 + 15) fraction under current of air to 2 mL, add 1 mL 2% alc. KOH, attach micro-Snyder column, and carefully reduce to ≤1 mL on steam bath. Reflux sample 15 min, remove, and cool. Add 2 mL alcohol–H_2O (1 + 1) and 5 mL hexane, and shake 1 min. Centrf. to sep. layers. Transfer as much hexane layer as possible to second tube, using disposable Pasteur pipet, and repeat extn with 5 mL hexane. Conc. combined hexane to appropriate vol. for GLC analysis.

29.D07 *Gas-Liquid Chromatography*

See **29.018.**

Collaborative Study

Prepared samples of perch and cod in quart jars were frozen and sent to collaborators. Previous analysis of this fish indicated that background interference would not alter expected results for the fortified samples. Collaborators were also sent 10 sealed glass vials with unknowns in hexane solution, a vial labeled Solution A, and a vial specified to contain *p,p'*-DDT in hexane.

Preliminary instructions required collaborators to inject 5 μL of Solution A into the GLC system at the beginning of each day of analyses. These chromatograms were to be submitted to the Associate Referee without any attempt to identify residues. Solution A contained lindane, heptachlor, aldrin, heptachlor epoxide, dieldrin,

Table 1. Fat content (%) in fish

	Coll.						
Sample	1	2	3	4	5	6	7
Perch	3.8	3.5	3.7	5.3	3.8	3.1	3.6
Cod	0.15	0.15	0.16	0.41	0.12	0	0.14

endrin, and *p,p'*-DDT. Chromatograms submitted for Solution A were expected to be comparable to the chromatogram shown in Figure 331-A of PAM, Vol. I, used by FDA. Evaluation of the submitted chromatograms showed that all the laboratories satisfactorily met the requirements for GLC instrumentation needed for this study.

Before analyzing fortified samples, collaborators were asked to analyze a reagent blank, a sample of nonfortified cod, a sample of nonfortified perch, and the solution specified to contain the *p,p'*-DDT in order to obtain experience with the method. The solution containing *p,p'*-DDT was to be transferred directly to a prepared miniaturized Florisil column and the *p,p'*-DDT concentraton was to be determined. Collaborators obtaining between 1.5 and 2.5 μg/mL were to proceed to the analysis of fortified samples. Collaborators with results outside the specified limits were to contact the Associate Referee for further instructions. The test was designed to show whether a major problem existed in the technique for preparing the Florisil column or in the technique for preparing reproducible concentrations of standard solutions of *p,p'*-DDT. Results of 6 collaborators (1.60, 1.64, 1.79, 1.67, 1.56, and 1.70 μg/mL) were in close agreement with the known concentration of 1.64 μg/mL, whereas Collaborator 4 had a higher result (2.36 μg/mL).

The collaborators were instructed to determine the percent fat content of the nonfortified fish samples according to the method in which constant weight is obtained. Table 1 shows reasonably close agreement among the 6 collaborators, with considerably higher results for Collaborator 4. Results indicate that the method is satisfactory for determining whether the fat content of the fish exceeds 10%.

Vial contents were added to a weighed portion of fish and mixed to fortify samples. Collaborators were instructed to analyze the samples in a particular order so that the duplicates would be run at different times. The collaborators were not informed that duplicates were included. The intent of the experimental design was to test the procedure for duplication on different days and also to see whether results improved with the analysts' experience.

Results

Table 2 shows the results for duplicate analyses of 5 fortified fish samples. Results were evaluated statistically in accordance with the *Statistical Manual of the AOAC* (4). The Youden rank sum test showed no outlying laboratories, i.e., laboratories which gave consistently high or consistently low results when compared with the other laboratories. Dixon's test applied residue by residue to the laboratory averages showed 2 outliers for Collaborator 2 and 2 outliers for Collaborator 4, as indicated in Table 2.

Table 3 presents the within-laboratory repeatability standard deviation (s_0), the overall between-laboratory reproducibility (s_x), and the corresponding coefficients of variation, (CV_0) and (CV_x). The CV_x values range from 6.3 to 20.6%. Two CV_x values exceed 16%, a number considered typical for a study of this nature.

Table 4 presents average amounts of recovered residues as compared with the amounts added for fortification, together with repeatability and reproducibility standard deviations. Average recoveries range from 79.3 to 100.7% of fortification levels with only 1 value above 100%.

Fortification levels of 0.49, 0.99, and 0.94 ppm in cod and 2.46, 0.99, and 2.35 ppm in perch for *p,p'*-DDE, *p,p'*-DDD, and *p,p'*-DDT, respectively, gave average recoveries exceeding 90% in all cases. Thus no apparent problems exist with the methodology that are due to various fortification levels or to the variance in the fat content of cod and perch. Cod fortified with 0.76 ppm Aroclor 1254 and perch fortified with 1.46 ppm Aroclor 1260 gave average recoveries of 100.7 and 86.6%, respectively, indicating that the method is satisfactory to determine PCBs. Separate analyses of cod and perch samples, each fortified with 0.20 ppm heptachlor epoxide and 0.15 ppm dieldrin, gave average recoveries of 88.5 and 79.3%, respectively. The results at these fortification levels should ensure detection of organochlorine pesticides and PCBs in fish at and below toler-

Table 2. Collaborative results for fortified fish samples (ppm)

	Soln 1, perch			Soln 2, cod			Soln 3, perch and cod		Soln 4, cod	Soln 5, perch
Coll.	*p,p'*-DDE	*p,p'*-DDD	*p,p'*-DDT	*p,p'*-DDE	*p,p'*-DDD	*p,p'*-DDT	Dieldrin	Hept. epox.	Aroclor 1254	Aroclor 1260
1	0.50	1.06	0.87	2.52	1.04	2.03	0.12	0.20	0.55	1.36
	0.50	0.97	0.85	2.88	0.90	1.88	0.13	0.18	0.67	1.44
2	0.44	0.82	0.81	2.72	1.35[a]	2.52	0.11	0.18	0.64	1.35
	0.47	0.84	0.90	2.61	1.13[a]	2.42	0.13	0.18	0.76	1.41
3	0.42	0.88	0.83	2.09	0.95	2.18	0.11	0.16	0.74	1.31
	0.44	0.91	0.92	2.16	0.97	2.16	0.13	0.16	0.79	1.31
4	0.50	1.03	0.88	2.34	1.13	2.66	0.11	0.18	0.85	0.69[a]
	0.45	0.91	0.75	1.97	0.71	1.74	0.11	0.16	0.95	1.11[a]
5	0.40	0.80	0.80	2.21	0.84	2.34	0.12	0.17	0.72	1.27
	0.41	0.82	0.83	2.15	0.80	2.26	0.12	0.18	0.74	1.19
6	0.53	1.00	0.98	2.11	0.81	1.87	0.12	0.21	0.95	1.44
	0.42	0.83	0.81	2.01	0.86	2.09	0.12	0.17	0.57	1.19
7	0.47	1.10	0.81	2.45	1.06	1.95	0.11	0.18	1.09	1.31
	0.49	1.15	0.84	2.21	0.92	1.75	0.12	0.17	0.69	1.31
Added	0.49	0.99	0.94	2.46	0.99	2.35	0.15	0.20	0.76	1.46

[a] Outliers by Dixon's test.

ance levels set by regulatory agencies of the United States and Canada.

Discussion

GLC analyses of the fish used in this study showed no residues before fortification or background interference, except Collaborator 1 reported 0.14 ppm Aroclor 1254. The fish was assumed to be free of residues in all cases, and the reported results are not corrected for any background.

Several collaborators reported that minor amounts of some organochlorine pesticides unexpectedly eluted from Florisil in the 15% ethyl ether-in-petroleum ether fraction.

Results are reported for the total of both fractions where possible. At least 97% of the reported values were for residues found in the proper fraction. The performance of the miniaturized Florisil column, as shown by the separation characteristics of the residues, was considered equivalent to that of the Florisil column described in PAM.

Recovery information was desired for dieldrin, which is often found as a residue in fish samples. Past experience showed that before GLC determination additional cleanup might be required for the 15% ethyl ether eluate containing the dieldrin, especially from fish samples with high fat (>5%) content. Instructions contained a disguised design whereby perch containing 0.15 ppm dieldrin were analyzed with the optional cleanup step given in the method and cod containing 0.15 ppm dieldrin were analyzed without additional cleanup. Recovery for 7 results obtained without additional cleanup averaged 82% compared with 76% for 7 results obtained after additional cleanup. The Associate Referee considers the results sufficiently significant to suggest that the additional cleanup step may be used as an option in place of the PAM additional cleanup. It is suggested that additional cleanup be routinely used for fish containing more than 5% fat.

Collaborator 6 reported an additional investigation in which a 50% ethyl ether eluant was used after the 15% ethyl ether eluant to check total elution of dieldrin and endrin. He found some dieldrin in the 50% fraction and suggested that a 50% ethyl ether eluant be included. The Associate Referee appreciates the suggestion and recommends it as an option, inasmuch as the average percent recovery for dieldrin was lower than the average percent recoveries for all other residues.

Table 3. Measures of precision for collaborative results

Sample	Residue	Added, ppm	Av. rec., ppm	s_0[a]	s_x[b]	CV_0,[c] %	CV_x,[d] %
1	*p,p'*-DDE	0.49	0.460	0.0342	0.0406	7.4	8.8
2	*p,p'*-DDE	2.46	2.316	0.1592	0.2863	6.9	12.4
3	*p,p'*-DDD	0.99	0.937	0.0630	0.1185	6.7	12.7
4	*p,p'*-DDD	0.99	0.962	0.1385	0.1702	14.4	17.7
			0.916[e]	0.1354[e]	0.1354[e]	14.8[e]	14.8[e]
5	*p,p'*-DDT	0.94	0.849	0.0677	0.0677	8.0	8.0
6	*p,p'*-DDT	2.35	2.132	0.2638	0.2861	12.4	13.4
7	Dieldrin	0.15	0.119	0.0085	0.0085	7.1	7.1
8	Heptachlor epoxide	0.20	0.177	0.0136	0.0144	7.7	7.7
9	Aroclor 1254	0.76	0.765	0.1572	0.1572	20.6	20.6
10	Aroclor 1260	1.46	1.264	0.1350	0.1946	10.7	15.4
			1.324[e]	0.0811[e]	0.0833[e]	6.1[e]	6.3[e]

[a] s_0 is within-laboratory repeatability standard deviation.
[b] s_x is overall between-laboratory reproducibility.
[c] CV_0 is coefficient of variation corresponding to s_0.
[d] CV_x is coefficient of variation corresponding to s_x.
[e] Outlying laboratories are excluded from result.

Table 4. Measures of precision for collaborative results

Sample	Residue	Av. rec., %	s_0[a]	s_x[b]
1	*p,p'*-DDE	93.9	6.98	8.29
2	*p,p'*-DDE	94.1	6.47	11.64
3	*p,p'*-DDD	94.6	6.36	11.97
4	*p,p'*-DDD	97.2	13.99	17.19
		92.5[c]	13.68[c]	13.68[c]
5	*p,p'*-DDT	90.3	7.20	7.20
6	*p,p'*-DDT	90.7	11.22	12.17
7	Dieldrin	79.3	5.67	5.67
8	Heptachlor epoxide	88.5	6.80	7.20
9	Aroclor 1254	100.7	20.68	20.68
10	Aroclor 1260	86.6	9.24	13.32
		90.7[c]	5.55[c]	5.71[c]

[a] s_0 is within-laboratory repeatability standard deviation.
[b] s_x is overall between-laboratory reproducibility.
[c] Outlying laboratories are excluded from result.

Recommendation

The Associate Referee believes that the within-laboratory repeatability and the between-laboratory reproducibility are satisfactory on the basis of the statistical evaluations of the collaborators' data. It is recommended that the method be adopted official first action.

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FISH AND OTHER MARINE PRODUCTS

Identification of Pacific Rockfish (*Sebastes*) Species by Isoelectric Focusing

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Isoelectric focusing (IEF) is currently the most reliable method available for the identification of fish species. The high resolution of this method usually allows discrimination between even closely related species. One genus, the *Sebastes*, does present a problem however. Using both low and high resolution, IEF is unable to differentiate several species. Disc electrophoresis, used in an AOAC official final action method, does not differentiate the rockfish reliably. Using IEF, identical protein patterns were obtained for Pacific Ocean perch (*Sebastes alutus*), Bocaccio rockfish (*S. paucispinis*), and yelloweye rockfish (*S. ruberrimus*). A second group, comprised of silvergray rockfish (*S. brevispinis*), yellowtail rockfish (*S. flavidus*), black rockfish (*S. melanops*), and canary rockfish (*S. pinniger*), also has identical protein patterns. Widow rockfish (*S. entomelas*) and chilipepper rockfish (*S. goodei*) each had a unique pattern, different from the above 2 groups and from each other. The actual taxonomic relationships of these rockfish species are not clear and further work with IEF may help in this regard. Users of IEF and disc electrophoresis for identification purposes should be aware of this problem when working with the *Sebastes*.

Isoelectric focusing (IEF) is currently the most reliable method available for the identification of fish species (1). IEF resolves many more protein bands than do other electrophoretic methods and is usually able to differentiate even closely related species. This is often an asset, but the resolution is so great that differences among individuals of the same species may become apparent. Monkfish (*Lophius americanus*) was the first species found to have such a polymorphic protein pattern with IEF. Six different monkfish protein patterns were found using polyacrylamide gel IEF and up to 16 different patterns could be resolved using high resolution IEF gels (2). Polymorphic protein patterns have also been described for black cow tongue (*Rhinoplagusia japonica*), black sea bream (*Mylio macrocephalus*), gizzard shad (*Konosirus punctatus*), frigate mackerel (*Auxis tapeninosoma*), carp (*Cyprinus carpio*), and *Tilapia nilotica* (3, 4). Both inter- and intraspecific variation has been found in sarcoplasmic proteins of whitefish, using IEF. Polymorphic patterns were reported for *Coregonus rasu*, *C. lauaretus*, *C. muksum*, *C. peled*, and *C. alhula*. Overall pattern variation was used to provide guidelines to the confusing taxonomy of the Coregonids (5).

Polymorphic protein patterns do not prevent accurate identifications by IEF provided the analyst is aware of the extent of variation to be expected within the species being examined. The opposite problem, more than one species having the same protein pattern, is of greater concern. Fish belonging to the genera *Thynnus* and *Tetrapturus* have been found to have sarcoplasmic protein patterns too similar to differentiate individual species by IEF (3). Before IEF came into use for fish species identification in the 1970s, it was found that disc electrophoresis could not differentiate several members of the *Sebastes*, the rockfish (6).

The family Scorpaenidae (rockfish and scorpionfish) form one of the most important groups of food fish found in the northeastern Pacific Ocean. There are more species in this one family of fish than any other (7). The classification of the *Sebastes* (formerly *Sebastodes*) using conventional morphological techniques is difficult. Many morphological characteristics have overlapping ranges among the various species. In an attempt to clarify the species distinctions, Tsuyuki et al. (8) compared sarcoplasmic protein patterns of 9 *Sebastes* and one *Sebastolobus* species using starch gel electrophoresis. They were unable to differentiate northern rockfish (*Sebastes polyspinis*) and dusky rockfish (*S. ciliatus*) by their protein patterns. In addition, each species showed 2 types of patterns. Northern and dusky rockfish are separated morphologically because

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Mention of trade names or commercial firms does not constitute endorsement by the National Marine Fisheries Service.

the former has 14 dorsal fin spines while the latter has only 13. They suggested that these fish may be a single species. Later, Tsuyuki et al. (9) compared sarcoplasmic protein and hemoglobin patterns from 26 *Sebastes* and 2 *Sebastolobus* species. Each *Sebastolobus* species had a unique protein pattern which differed from any of the *Sebastes* patterns. The *Sebastes*, however, had many species with identical patterns. They were able to divide the *Sebastes* into 4 groups, each of which had a distinctive pattern type. The hemoglobin patterns were more specific, which allowed the *Sebastes* to be further subdivided. We have found that despite the higher resolving power of IEF, there are still several *Sebastes* species which have virtually identical sarcoplasmic protein patterns and thus cannot be differentiated on this basis. Presented in this paper are sarcoplasmic protein patterns from 9 *Sebastes* and 2 *Sebastolobus* species separated by IEF and disc electrophoresis.

Materials and Methods

The species used in this study were obtained from various processors in California, Oregon, and Washington. The whole fish were identified by fishery biologists before shipment on wet ice to Gloucester, MA, via air freight. On arrival, identifications were confirmed by reference to the key by Hitz (10). The fish were then frozen at −18°C until analysis. From 1 to 4 individuals from each species were tested.

The species used in this study were: (*1*) Pacific ocean perch (*Sebastes alutus*); (*2*) silvergray rockfish (*S. brevispinis*); (*3*) widow rockfish (*S. entomelas*); (*4*) yellowtail rockfish (*S. flavidus*); (*5*) chilipepper rockfish (*S. goodei*); (*6*) black rockfish (*S. melanops*); (*7*) Bocaccio rockfish (*S. paucispinus*); (*8*) canary rockfish (*S. pinniger*); (*9*) yelloweye rockfish (*S. ruberrimus*); (*10*) shortspine thornyhead (*Sebastolobus alascanus*); (*11*) longspine thornyhead (*Sebastolobus altivelis*).

All scientific and common names are those recognized by the American Fisheries Society (11).

Polyacrylamide gel IEF was performed according to the AOAC official final action method based on LKB Ampholine PAG plates (12).

Agarose gel IEF was performed according to Lundstrom (13) except 1.5 mL of pH 3.5–9.5 or pH 5.0–8.0 ampholine was substituted for the ampholine mixture stated. Disc electrophoresis was performed according to the AOAC official final action disc electrophoresis method (14) except that the proteins were fixed and stained as in the agarose gel IEF method (13) and destaining was accomplished by diffusion rather than by electrophoresis.

Results and Discussion

All rockfish of the genus *Sebastes* have the following characteristics, which differentiate them from related groups: (*1*) a suborbital bony stay; (*2*) thoracic ventral fins with 1 spine and 5 soft fin rays; (*3*) gill openings extending to at least the lowest pectoral fin ray; (*4*) a uniform scaled body; (*5*) a reduced or absent slit posterior to the fourth gill arch; (*6*) 2 spines on the upper rear corner of the gill cover; (*7*) 5 short spines on the rear edge of the preopercle; (*8*) 13 dorsal fin spines (11–14 rarely); (*9*) 11–18 soft dorsal rays; (*10*) 26 or 27 vertebrae.

Among individuals with these characteristics, the species are divided by their color, number and location of head spines, length of anal spines, head shape, and peritoneum color (10).

Chu (6), using disc electrophoresis, compared the sarcoplasmic protein patterns of several rockfish. He found identical protein patterns for striped tail rockfish (*Sebastes saxicola*), widow rockfish, blue rockfish (*S. mystinus*), olive rockfish (*S. serranoides*), and Pacific Ocean perch. Unique patterns were found for chilipepper and Bocaccio rockfish. Figure 1 shows the 9 species of rockfish tested using the AOAC disc electrophoresis method. All rockfish species tested had identical protein patterns, except the chilipepper rockfish. In contrast with Chu's results (6), a unique pattern for the Bocaccio rockfish could not be demonstrated.

It was hoped that the greater resolution of IEF would allow differentiation of the rockfish species. Figure 2 shows sarcoplasmic protein patterns, obtained using the AOAC polyacrylamide gel IEF method, for 2 *Sebastolobus* and 9 *Sebastes* species. The shortspine and longspine thornyheads each had a unique pattern, very distinct from any of the rockfish patterns. The second longspine thornyhead pattern shows what is probably a protein polymorphism: an additional band near the top of the gel which does not appear in the other individual of the same species. Additional individuals need to be tested to determine the nature and extent of this extra band. The widow and chilipepper rockfish each had a unique pattern which reproduced well between the duplicate samples. The remaining seven rockfish species had patterns that were very similar. A number of minor differences were noted among the 7 species but just as much variation was seen between duplicate samples of the same species. Polymorphic pro-

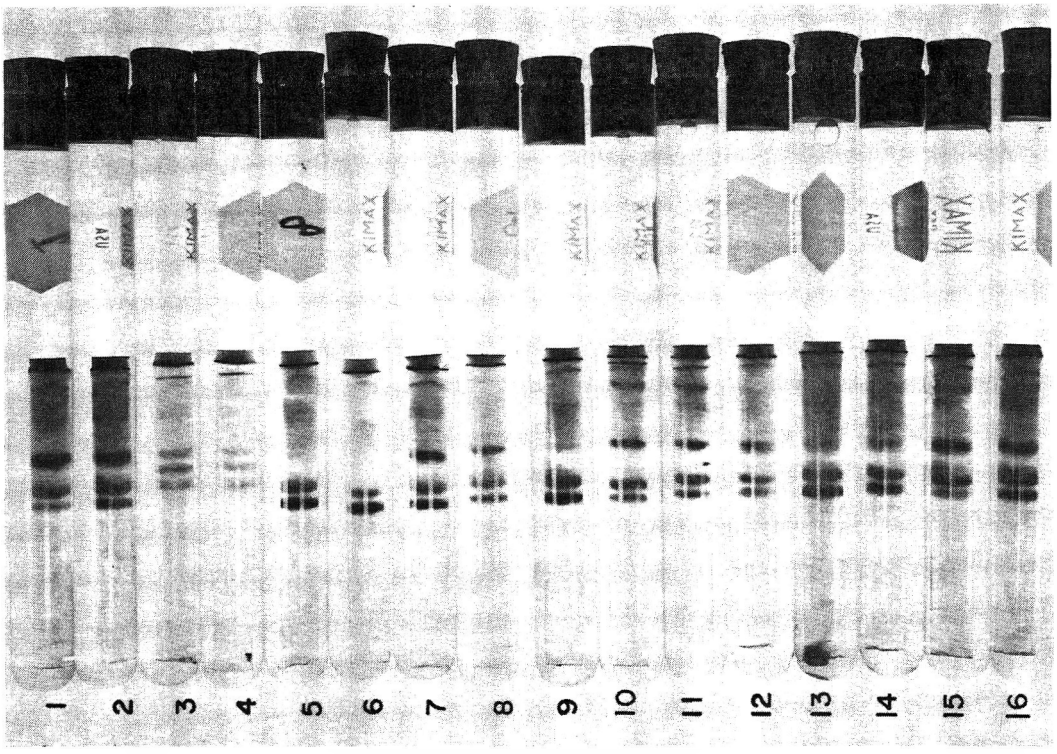

Figure 1. Sarcoplasmic protein patterns for 9 *Sebastes* species separated by disc electrophoresis. Individual samples are: 1 and 2 (widow rockfish); 3 and 4 (chilipepper rockfish); 5 and 6 (Pacific Ocean perch); 7 and 8 (Bocaccio rockfish); 9 (yelloweye rockfish); 10 and 11 (silvergray rockfish); 12 (yellowtail rockfish); 13 and 14 (black rockfish); 15 and 16 (canary rockfish).

teins in rockfish were seen frequently by Tsuyuki et al. using starch gel electrophoresis but they sampled a large enough number of individuals from each species to be assured of detecting any possible polymorphisms. Because we were unable to sample a large number of individuals, it was impossible to predict the number and extent of rockfish polymorphisms that could be detected using IEF.

Agarose gel IEF in a pH 3.5–9.5 (Figure 3) or pH 5.0–8.0 (Figure 4) gradient shows essentially the same results as obtained using the polyacrylamide gel IEF method (Figure 2). The same polymorphism is seen with the longspine thornyhead samples, and the widow and chilipepper rockfish were the only rockfish species showing unique patterns.

Figure 5 shows another pH 5.0–8.0 agarose gel IEF experiment in which the 2 thornyhead and 9 rockfish species were compared. On this gel, 5 times the normal sample load was used so that some of the minor protein bands could be examined. With the larger sample, an additional protein is resolved just below the darkest top band in the Pacific Ocean perch, and Bocaccio and yelloweye rockfish samples. Thus, these 3 species can be differentiated from the silvergray, yellowtail, black, and canary rockfish samples, all of which lack this additional band.

The Pacific rockfish present a general problem in speciation using protein banding patterns. Starch gel and disc electrophoresis and IEF are unable to differentiate several rockfish species. Chu stated that the rockfish sarcoplasmic protein patterns obtained by disc electrophoresis were distinct from those of other species (6). We find the same to be true of IEF. An analyst faced with the need to identify an unknown sample would be able to determine if it were a rockfish. If it did appear to have the characteristic rockfish pattern, speciation would only be possible if the unknown were widow or chilipepper rockfish. Using a large sample load and a narrow range carrier ampholyte (high resolution), one should be able to further decide if the unknown was a member of one of the rockfish groups differentiated as in Figure 5.

It is difficult to make recommendations based on a limited study such as this. Certainly these preliminary results suggest that additional

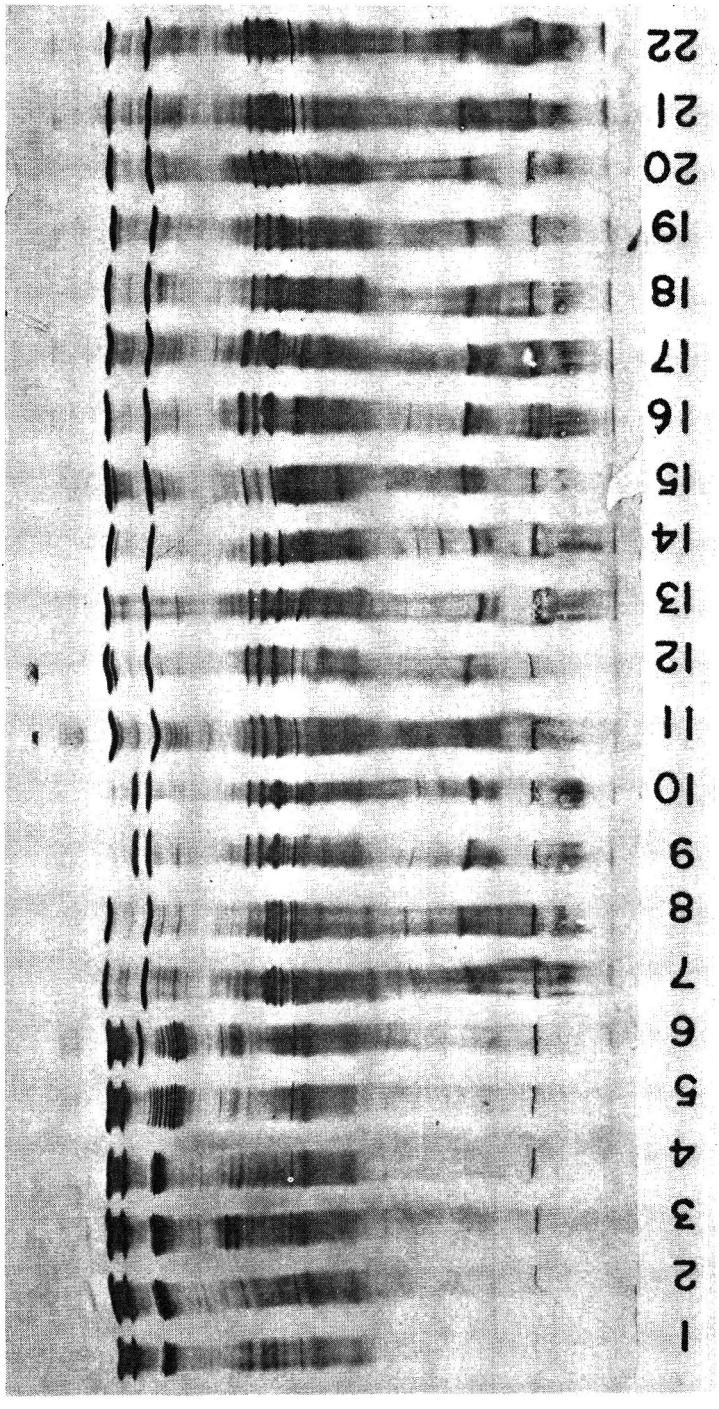

Figure 2. Sarcoplasmic protein patterns for 2 *Sebastolobus* and 9 *Sebastes* species separated on a pH 3.5–9.5 polyacrylamide IEF gel. Species are: 1-4 (shortspine thornyhead); 5 and 6 (longspine thornyhead); 7 and 8 (widow rockfish); 9 and 10 (chilipepper rockfish); 11 and 12 (Pacific Ocean perch); 13 and 14 (Bocaccio rockfish); 15 (yelloweye rockfish); 16 and 17 (silvergray rockfish); 18 (yellowtail rockfish); 19 and 20 (black rockfish); 21 and 22 (canary rockfish).

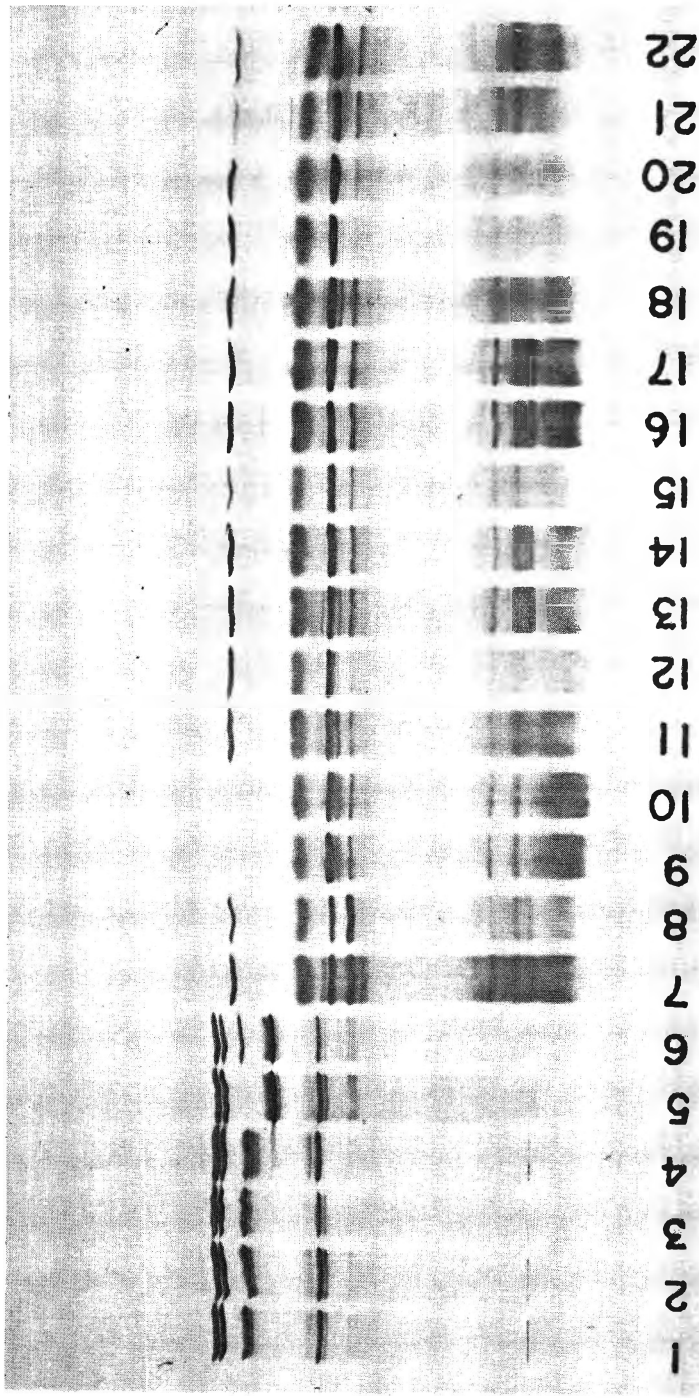

Figure 3. Sarcoplasmic protein patterns for 2 *Sebastolobus* and 9 *Sebastes* species separated on a pH 3.5–9.5 agarose IEF gel. Order of samples same as in Figure 2.

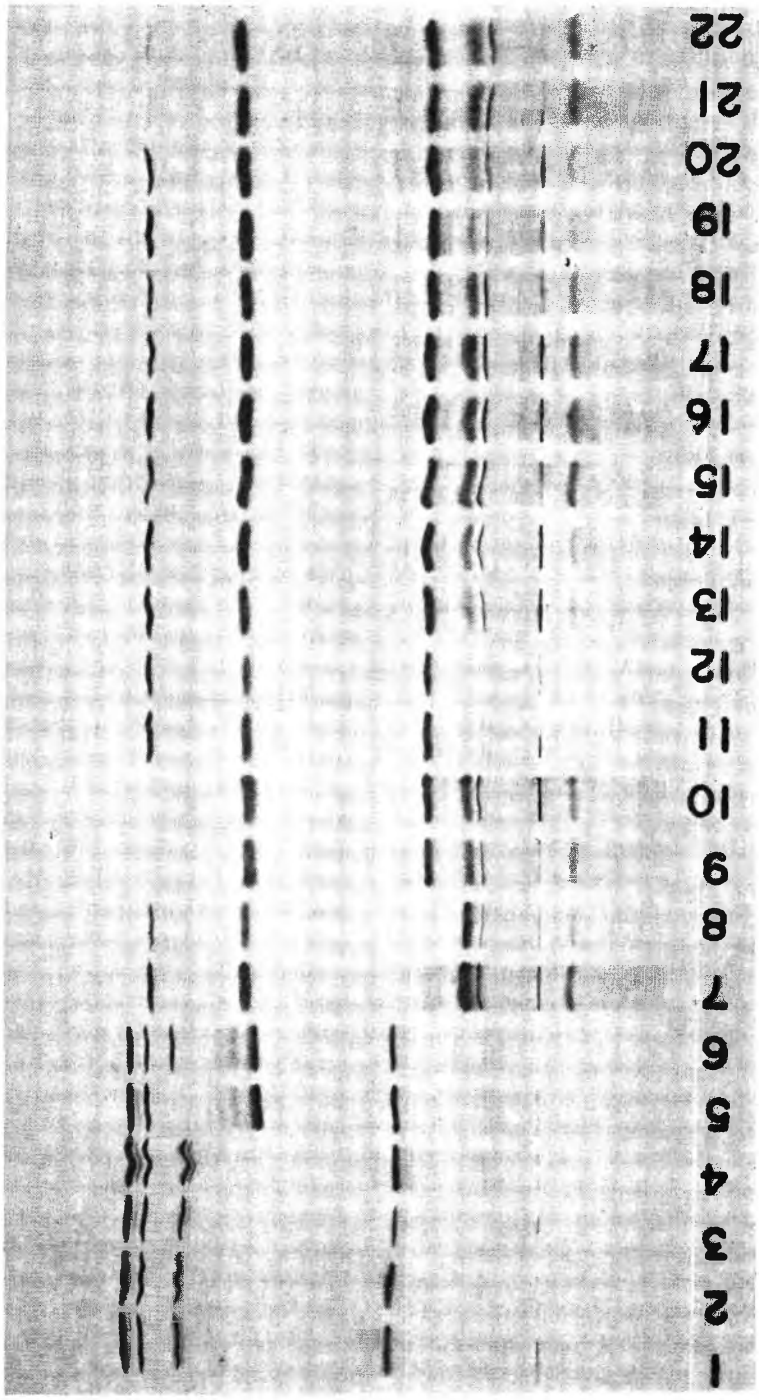

Figure 4. Sarcoplasmic protein patterns for 2 *Sebastolobus* and 9 *Sebastes* species separated on a pH 5.0–8.0 agarose IEF gel. Order of samples same as in Figure 2.

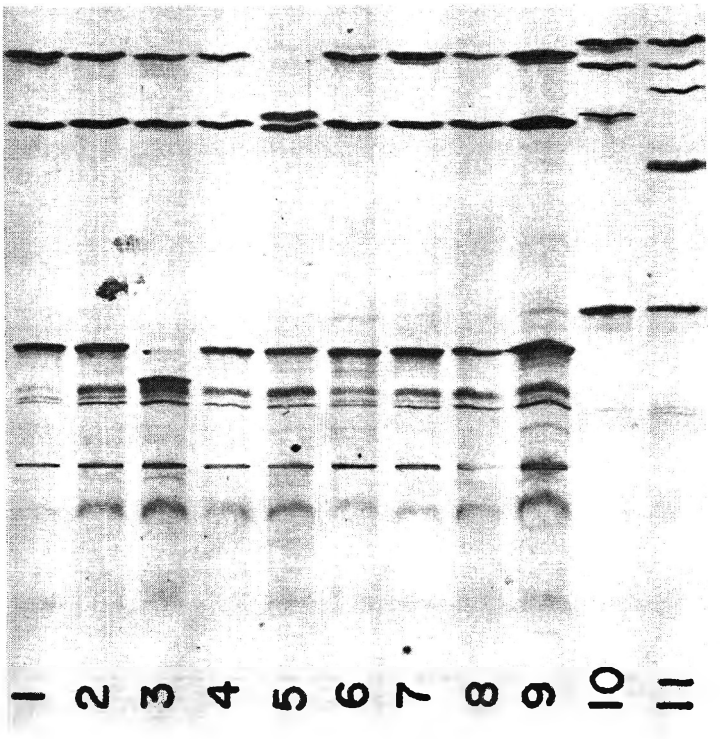

Figure 5. Sarcoplasmic protein patterns for 9 *Sebastes* and 2 *Sebastolobus* species separated on a pH 5.0-8.0 agarose IEF gel. Five times the normal sample load was used to visualize minor components. Order of species is: 1 (Pacific Ocean perch); 2 (silvergray rockfish); 3 (widow rockfish); 4 (yellowtail rockfish); 5 (chilipepper rockfish); 6 (black rockfish); 7 (Bocaccio rockfish); 8 (canary rockfish); 9 (yelloweye rockfish); 10 (shortspine thornyhead); 11 (longspine thornyhead).

rockfish species and larger numbers of individuals need to be examined. Potential users of electrophoresis and IEF methods for fish species identification should be aware of this problem, and any rockfish identifications should be carefully considered.

Acknowledgments

The author thanks Frederick J. King (NMFS, Gloucester, MA) for procuring the rockfish samples used in this study and for help with the confusing taxonomy of this group of fish. The author also thanks Rebecca Marsden for expert technical assistance.

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SUGARS AND SUGAR PRODUCTS

Determination of Glucose, Sucrose, Lactose, and Ethanol in Foods and Beverages, Using Immobilized Enzyme Electrodes

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An enzyme electrode coupling immobilized oxidase enzymes with a hydrogen peroxide-sensitive electrode is described. An enzyme or enzymes are immobilized in a thin microporous membrane which fits directly over a platinum anode held at +0.700 V relative to a $Ag/AgCl_2$ reference electrode. When an enzyme substrate diffuses into the membrane, hydrogen peroxide is produced. The hydrogen peroxide is then oxidized at the platinum anode, producing an electrical current that is directly proportional to hydrogen peroxide concentration, and hence substrate concentration. Glucose and sucrose in cereal samples, lactose in cheese, and ethanol in beer and wine were determined using enzyme electrodes. Relative precision of replicate analyses was better than ±2% and agreement with AOAC methods was good.

Clark and Lyons (1) are usually credited with describing the first enzyme electrode. Since then, the use of immobilized enzyme electrodes as analytical tools has been increasing rapidly. Review articles describing the properties of these devices are available (2–4). In addition, enzyme electrodes have been reported for the determination of ethanol (5), glucose (6), galactose (7), and other substances. This paper describes an amperometric enzyme electrode where oxidase enzymes are coupled with a hydrogen peroxide-sensitive electrode. This system is commercially available for determining glucose, sucrose, lactose, starch, or ethanol.

Experimental

Enzyme Electrode

Figure 1 is an enlargement of the enzyme electrode. The patented membrane (8) covering the electrode has 3 layers. The outermost layer is a thin (~5 μm) polycarbonate film with pores of ~0.03 μm diam. Behind this film is the immobilized enzyme(s). This paper discusses immobilized enzyme membranes that are currently available: glucose membranes (glucose oxidase), sucrose membranes (invertase, mutarotase, glucose oxidase), lactose membranes (galactose oxidase), and alcohol membranes (alcohol oxidase). When an enzyme substrate diffuses through the outer layer of the membrane and encounters the enzyme layer, hydrogen peroxide is produced (Figure 2). The hydrogen peroxide diffuses through the cellulose acetate layer where it is oxidized at the platinum anode. This oxidation produces an electrical current directly proportional to hydrogen peroxide concentration, and hence to substrate concentration. Other oxidizable substances that reach the electrode would contribute to a high reading. The cellulose acetate layer, with a molecular weight cutoff at ~100, prevents most such interferences from reaching the electrode.

Apparatus

(a) *Model 27 industrial analyzer.*—Equipped with immobilized enzyme membranes (Yellow Springs Instrument Co., Scientific Division, Box 279, Yellow Springs, OH 45387).

(b) *Syringepet.*—Combination syringe and pipet (Yellow Springs Instrument Co.).

Reagents

All reagents used for standards and buffers were ACS reagent grade.

Standards

All standards were prepared in deionized water containing 0.1% K_2EDTA as a preservative. These standards are stable for 6 months at room temperature.

Procedure

Glucose and sucrose.—When glucose and sucrose are present in the same sample, the sucrose membrane previously described cannot be used because its response would be a weighted sum of the glucose and sucrose present (see Figure 2). Sucrose can be determined in the presence of glucose by measuring the glucose before and after the hydrolysis of sucrose. We assayed 4 cereal samples by using the enzyme electrode

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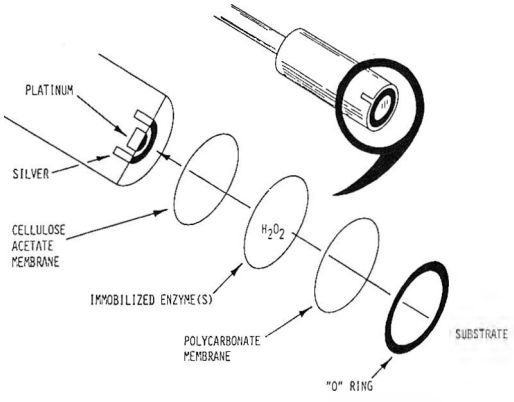

Figure 1. Diagram of enzyme electrode.

method. Approximately 2.00 g finely ground cereal was stirred with ca 75 mL 0.25M phosphate buffer at room temperature for 15 min. This mixture was transferred to a 100 mL volumetric flask and diluted to volume with buffer. Glucose was determined by injecting a 25 μL aliquot (with a Syringepet) into a calibrated Model 27 analyzer (6). The readout, in mg glucose/100 mL, was multiplied by the appropriate dilution factor to determine glucose concentration in the sample.

To determine sucrose concentration, ca 2 mg invertase was added to a 2 mL aliquot of the sample dilution. This was allowed to incubate for 30 min at room temperature and then assayed

Glucose:

$$\beta\text{-D-glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} H_2O_2 + \text{Gluconic Acid}$$

Sucrose:

$$\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \text{Fructose} + \alpha\text{-D-glucose}$$

$$\alpha\text{-D-glucose} \rightleftharpoons \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} H_2O_2 + \text{Gluconic Acid}$$

Lactose:

$$\text{Lactose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Galactose}} H_2O_2 + \text{Oxidation Product}$$

Ethanol:

$$\text{Ethanol} + O_2 \xrightarrow[\text{Oxidase}]{\text{Alcohol}} H_2O_2 + \text{Acetaldehyde}$$

H_2O_2:

$$H_2O_2 \xrightarrow[\text{Anode}]{\text{Pt}} 2H^+ + O_2 + 2e^-$$

Figure 2. Enzymatic reactions leading to production of H_2O_2 which is measured electrochemically at the platinum anode.

Table 1. Sucrose and dextrose concentrations (%) in 4 cereals by enzyme electrode and HPLC methods

Sample	Enzyme electrode[a] Sucrose ±SD	Enzyme electrode[a] Dextrose ±SD	HPLC[b] Sucrose	HPLC[b] Dextrose
A	35.0 ± 0.6	1.24 ± 0.05	36.0	0.96
B	22.4 ± 0.4	1.52 ± 0.06	23.1	1.52
C	21.4 ± 0.4	0.85 ± 0.03	22.2	0.50
D	5.40 ± 0.09	1.03 ± 0.03	6.70	0.70

[a] Quadruplicate analyses.
[b] AOAC method **14.C01–14.C04.**

Table 3. Ethanol concentration (% v/v) in 8 wine samples by enzyme electrode and AOAC methods

Sample	Enzyme electrode ±SD (n = 6)	AOAC **11.005**
A	6.71 ± 0.05	6.85
B	7.38 ± 0.07	7.34
C	10.62 ± 0.06	10.61
D	10.71 ± 0.08	10.78
E	11.07 ± 0.07	11.03
F	11.44 ± 0.12	11.70
G	11.71 ± 0.16	11.70
H	12.14 ± 0.07	12.12

for glucose. The glucose value after hydrolysis minus the initial glucose multiplied by 1.9 yields sucrose concentration. The 1.9 factor is used because 1.9 g sucrose yields 1.00 g glucose on hydrolysis.

These 4 samples were also assayed by the official first action AOAC HPLC method for mono- and disaccharides in presweetened cereals (9).

Lactose.—Lactose can be determined in a number of samples by the enzyme electrode method. Because galactose oxidase is the enzyme used, it is important that the sample be free of other galactosides that may be substrates of this enzyme. We assayed 3 processed cheese samples by diluting the cheese in buffer as previously described for cereal samples. Lactose in these samples was also determined by high pressure liquid chromatography (HPLC).

Ethanol.—The measurement of ethanol requires alcohol oxidase. Methanol is also a substrate for this enzyme, but is generally only present in trace amounts in samples of interest. We have described measurement of ethanol in beer by using enzyme electrodes (10). Samples are prepared by simple dilution.

The enzyme electrode procedure was also used for ethanol determination in wine. AOAC specific gravity methods (11, 12) were used as a reference.

Table 2. Lactose concentration (%) in 3 cheese samples by enzyme electrode and HPLC methods

Sample	Enzyme electrode[a] ± SD	HPLC[b]
A	9.81 ± 0.12	10.0
B	7.52 ± 0.10	7.80
C	10.1 ± 0.17	9.70

[a] Quadruplicate analyses.
[b] AOAC method **14.C01–14.C04.**

Results

Results of glucose and sucrose analyses of cereal samples are shown in Table 1. There is good agreement between the 2 methods and the reproducibility of the enzyme electrode procedure is excellent.

Results of lactose analyses are shown in Table 2. Again, there is good agreement with the HPLC analysis, and good reproducibility.

Tables 3 and 4 summarize our results on wine and beer analysis using enzyme electrodes. These analyses also demonstrate the accuracy and precision of enzyme electrodes.

Discussion

The combination of immobilized enzymes with electrochemical detectors offers the analytical chemist an extremely powerful tool for the determination of simple substances in complex matrices. The above analyses, even though run in only a semiautomated instrument, were performed much more rapidly than more classical techniques. While the reference methods in this work were not repeated, the general precision of these methods has been reported in the literature and the enzyme electrode procedure has shown

Table 4. Ethanol concentration (% v/v) in beer samples by enzyme electrode and AOAC methods

Sample	Enzyme electrode ±SD (n = 6)	AOAC **10.023**
A	4.30 ± 0.04	4.26
B	4.35 ± 0.03	4.26
C	4.50 ± 0.02	4.48
D	4.50 ± 0.05	4.40
E	4.70 ± 0.03	4.77
F	4.75 ± 0.03	4.69
G	4.76 ± 0.03	4.77
H	4.90 ± 0.06	4.84
I	5.40 ± 0.06	5.51
J	5.55 ± 0.05	5.51

similar accuracy and precision. Analysis time after dilution is approximately 2 min. Reagent cost is less than $0.30/test. The specificity of this technique allows minimal sample preparation, thereby minimizing a major source of error in most analytical techniques.

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MEAT AND MEAT PRODUCTS

Determination of Lead in Bonemeal by Differential Pulse Anodic Stripping Voltammetry Using a Hydrochloric Acid Solubilization

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A safe, rapid method is described for the determination of lead in bonemeal. This method uses a hydrochloric acid solubilization performed under pressure followed by determination by differential pulse anodic stripping voltammetry. This provides an alternative to a nitric-perchloric acid wet ash. Data obtained using both methods are compared. The mean recovery of a standard Pb spike was 99.2 ± 7%. The concentration of Pb in bonemeal ranged from 1.0 to 15.6 μg/g.

Bonemeal is a supplemental source of calcium and phosphorus for human consumption. It is produced by crushing cow bone to a fine powder and is marketed as a powder or tablet. It is often added, along with red bone marrow, to multiple vitamins. Bone incorporates Pb(II) as a biological defense mechanism, thus removing it from other cellular processes (1). Consequently, the level of Pb in bone increases with the age of the animal.

Health authorities are concerned that prolonged exposure of children and pregnant women to Pb in bonemeal could cause adverse health effects. The current method (2) for the determination of Pb in this material uses a $HNO_3/HClO_4$ wet ash. Inconsistent replicate determinations by the wet ash method in this laboratory have prompted the investigation of an alternative procedure.

The methodology presented here combines an HCl solubilization performed under pressure and increased temperature with differential pulse anodic stripping voltammetry (DPASV). The result is a sensitive method for Pb in bonemeal with greatly reduced sample pretreatment.

METHOD

Apparatus

(a) *Polarograph.*—PAR Model 384 polarographic analyzer or equivalent, equipped with Model 303 static mercury dropping electrode and Model 305 stirrer (Princeton Applied Research, Princeton, NJ 08540).

(b) *X-Y recorder.*—Hi-Plot digital plotter (Houston Instruments, Austin, TX 78753), or equivalent.

(c) *Electrolyte purification apparatus.*—PAR Model 9500, or equivalent.

(d) *Shaker.*—Rotary shaker (Fermentation Design, Inc., Allentown, PA 18103, or equivalent).

(e) *Oven.*—Forced convection oven (Hotpack, Philadelphia, PA 19154, or equivalent).

(f) *Funnels.*—Polypropylene, Buchner type, 43 mm diameter (Mallinckrodt, Inc., Raleigh, NC 27601), or equivalent.

(g) *Teflon filters.*—Mitex type LS, 47 mm diameter, pore size 5.0 μm (Millipore Corp., Bedford, MA 01730).

(h) *Solubilization bottles.*—Wide mouth, 60 mL linear polyethylene (LPE) (Nalge Company, Box 365, Rochester, NY 14602), or equivalent.

Reagents

(a) *Distilled deionized water (DDW).*—18 Megohm cm^{-1} (Millipore Corp., Bedford, MA 01730).

(b) *Hydrochloric acid.*—"Instra Analyzed" (J.T. Baker Chemical Co., Phillipsburg, NJ 08865).

(c) *Buffer/electrolyte.*—pH ca 4.6. Dissolve 102 g anhydrous sodium acetate, 8 g tartaric acid, and 97 mL glacial acetic acid, ACS reagent grade, in 500 mL DDW and dilute to 1.0 L with DDW. A potential of −1.2 V vs an SCE applied to this stirred solution for 4 days should reduce the Pb level below 80 pg/mL.

(d) *Lead standard solution.*—10 μg/mL. Prepare daily from 1000 μg/mL stock lead standard (Spex Industries, Inc., Metuchen, NJ 08840).

Preparation and Determination

Perform all sample preparation steps in clean air environment while wearing disposable polyethylene gloves. Pulverize tablet samples with acid-washed glass mortar and pestle and store in acid-washed LPE bottles.

Accurately weigh 2–3 g duplicate samples into

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Table 1. Comparison of duplicate analyses and recoveries using HCl solubilization [a] and $HNO_3/HClO_4$ wet ash techniques

Sample	HCl, μg/g	Rec., %	$HNO_3/HClO_4$, μg/g	Rec., %
1	7.1	96	7.7	100
	7.1		7.8	
2	16	102	14	89
	18		16	
3	6.0	92	6.2	87
	7.0		7.3	
4	7.6	105	7.8	92
	8.8			
5	3.4	92	3.9	94
	4.7		4.5	
6	14	102	11.6, 12.0, 13.0	110
	15		13.8, 13.9, 17.6	
7	7.7	94	5.7, 6.3, 7.1	71
	9.0		7.6, 8.7, 9.8	

[a] 3M HCl solution in electrochemical cell.

acid-washed LPE bottles. Add 38 mL 6M HCl to each sample, cap bottles tightly by hand, and place on rotary shaker (200 rpm) located inside the oven at 85°C for 30 min. Transfer cooled sample from LPE bottle to 100 mL volumetric flask with DDW and dilute to volume. (Precipitation of collagenous materials is normal with change in pH.) Vacuum-filter portion of sample solution, using Teflon filter with polypropylene Buchner funnel.

Transfer 5.0 mL sample solution to polypropylene cell containing 5.0 mL DDW (final acid concentration ca 1.1M). Transfer Teflon stirring bar (12.7 × 3 mm) to cell and clamp cell in place over stirring motor. Purge solution with oxygen-free nitrogen saturated with electrolyte for 5 min initially and 30 s between successive runs. Set deposition potential at −0.670 V; deposition time 60 s with stirring; equilibration time 15 s; pulse modulation 50 mV; scan rate 4 mV/s; final potential −0.320 V; current sensitivity commensurate with concentration; medium drop size; 400 rpm stirring rate. All measurements were made at 23 ± 0.5°C with Ag/AgCl reference electrode.

Quantitation

Quantitate analyte level, using method of additions of microliter volumes of standard to sample cell. Determine peak amplitudes (nA) with respect to tangent fitted to the base of peak. Calculate analyte concentration from resulting peak amplitude and corresponding amount of standard added (μg), using linear regression analysis. Analyte level (μg) is the absolute value of X-axis intercept. Calculate analyte concentration in the sample from following equation:

Analyte level (μg/g) = [(μg sample − μg blank) × dilution factor]/sample weight

Dilution factor is sample dilution volume divided by sample volume delivered to electrolysis cell.

Experimental

Recoveries were evaluated by fortifying the samples with stock Pb standard. The level of fortification depended on the Pb level in the samples and the weight of sample analyzed. Typically, 10–50 μg Pb was added to a sample before solubilization. The percent recovery was calculated by subtracting the average of duplicate unspiked results for a particular sample from the result for the spiked sample, dividing by the spike level (μg Pb added/g sample), and multiplying by 100. Percent recoveries are listed in Tables 1 and 2.

Results and Discussion

Initial experiments performed by using a 6M HCl solubilization on agricultural crop samples (3) demonstrated the feasibility of this technique for the extraction of trace elements. This solubilization procedure was evaluated for the determination of Pb in commercial bonemeal samples by comparison with the $HNO_3/HClO_4$ procedure. The sample digest was initially diluted to volume with 6M HCl. The results shown in Table 1 were obtained with a solution of 5 mL DDW and 5 mL sample, making the final acid concentration 3M.

The results compared favorably with the $HNO_3/HClO_4$ procedure. However, precision of duplicate analyses with final acid concentration was no better than with the $HNO_3/HClO_4$

Table 2. Comparison of HCl solubilization [a] and $HNO_3/HClO_4$ wet ash techniques in the analysis of bonemeal samples

Sample	HCl, μg/g	Rec., %	$HNO_3/HClO_4$, μg/g	Rec., %
6	15.5	98	11.6, 12.0, 13.0	110
	15.8		13.8, 13.9, 17.6	
7	6.9	92	5.7, 6.3, 7.1	71
	7.1		7.6, 8.7, 9.8	
8	1.01	98	1.06	84
	1.05		1.08	
9	1.00	93	1.04	—
	1.00		1.08	
10	10.2	—	9.1	—
	10.4		12.2	
11	8.8	104	9.2	113
	8.9		9.6	
12	1.3	92	0.66	83
	1.3		0.93	
13	3.9	109	4.0	81
	4.0		5.1	
14	3.3	107	4.0	82
	3.4		4.1	

[a] 1.1M HCl solution in electrochemical cell.

procedure. This may partially be due to the interference of a large Cu oxidation peak with the Pb stripping peak. The acid concentration was reduced from 3M to 1.1M by dilution of the solubilized sample with DDW rather than with 6M HCl. The lower acid concentration resulted in better resolution of Pb and Cu oxidation peaks and increased Pb sensitivity. Table 2 presents results of duplicate analyses obtained by this procedure and the $HNO_3/HClO_4$ procedure. Recoveries and precision of duplicate analyses with 1.1M HCl in the electrochemical cell show an improvement over the $HNO_3/HClO_4$ procedure. This indicates that sample homogeneity was not the problem which led to inconsistent duplicate analyses and points to a possible problem in the $HNO_3/HClO_4$ digestion procedure.

Samples 1–10 and 15 contained only bonemeal, either in powder or tablet form. Samples 11–13 were bonemeal tablets with vitamin D added. Sample 16 consisted of bonemeal tablets containing vitamins A and D. Sample 14 consisted of bonemeal tablets containing vitamin D, sugar, starch, ethylcellulose, stearic acid, and magnesium stearate. This product showed reduced sensitivity for added Pb along with an elevated background current. Although duplicate analyses of this sample demonstrated good precision, the Pb values were not in agreement with those obtained by using the $HNO_3/HClO_4$ wet ash.

The analysis of 4 reagent blanks resulted in a mean Pb level in the electrochemical cell (1.1M HCl) of 0.0058 μg. This is primarily due to the Pb level in the HCl and is considerably below the levels encountered in the samples.

One drawback to this technique is the coincidence of the Sn reduction peak with the Pb peak as shown in Figure 1. Both Sn and Pb are oxidizable from the Hg drop at the same potential. This is a common type of interference in DPASV (4–7). Comparison of the solubilization data with the $HNO_3/HClO_4$ data, which do not exhibit a Sn-Pb interference, indicates that none of the samples investigated demonstrated a contribution to the Pb level by Sn. However, the possibility of this interference had to be addressed. Jones and Brasker (5) suggested that a simple pH change to between 2.5 and 3.0 would be sufficient to shift the Sn reduction potential. This was accomplished by addition of NH_4OH to the electrochemical cell. However, addition of 20 μg of Sn to the cell produced a substantial increase in the Pb signal. Therefore, a buffered-tartaric acid system was chosen in which an electro-inactive Sn-tartrate complex was formed (6) with a 4:1 ratio of buffer to sample, which produced a solution pH of 3.9 ± 0.1. At this pH, Sn does not interfere even when 100 μg is added directly to the sample cell, which corresponds to 2,000 μg Sn in the original sample. A scan from −1.5 to −0.320 V showed no reduction-oxidation peak for the tartrate complex. In Table 3 the results obtained after bonemeal samples were spiked with Sn before solubilization were compared to those obtained without the addition of Sn. There was no difference in the analyte level in the presence of added Sn.

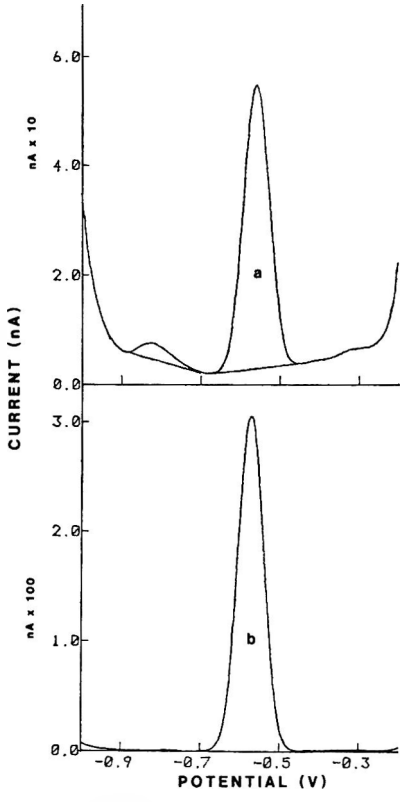

Figure 1. Differential pulse anodic stripping voltammograms of the coincidence of Pb and Sn stripping peaks: a, 0.1 μg Pb in 1.1M HCl, 60 nA scale; b, 5.0 μg Sn added to solution a, 300 nA scale.

The solution of 8 mL buffer and 2 mL sample resulted in decreased sensitivity due to the dilution. Instrumental parameters such as deposition time, pulse modulation, stirring rate, drop size, and scan rate can be adjusted to increase sensitivity. The analysis of 4 reagent blanks resulted in a mean Pb level in the electrochemical cell of 0.0029 μg contributed by the HCl.

Table 3. Tin interference data

Sample	No tin added,[a] μg Pb/g	200 μg tin added,[b] μg Pb/g
13	4.0	3.9
14	3.4	3.4
15	4.0	4.3
16	6.1	6.2

[a] 5 mL DDW + 5 mL sample.
[b] 8 mL buffer (2.1M HOAc/1.2M NaOAc/0.05M tartaric acid) + 2 mL sample.

Conclusions

The HCl solubilization method for determining Pb in bonemeal provides an accurate, precise alternative to the $HNO_3/HClO_4$ method. The HCl method reduces the danger associated with a perchloric acid digestion, requires less sample preparation time, and exhibits better precision. There is little probability of an interference from Sn in bonemeal at a level which affects the Pb determination; however, an alternative determinative step has been described which addresses this possibility.

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Titrimetric Determination of Calcium in Mechanically Separated Poultry and Beef: Collaborative Study

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A collaborative study was conducted on a titrimetric EDTA method for the determination of calcium in mechanically separated poultry and beef tissue. Six laboratories analyzed, as blind duplicates, 5 poultry samples and 5 beef samples. Calcium concentrations covered the levels from 0.04 to 0.82%. Pooled standard deviations were 0.0165 for repeatability, 0.0072 for bias, and 0.0225 for reproducibility. The method has been adopted official first action.

The growth of the poultry industry in the past 25 years created the need for technology to develop uses for surplus poultry parts, primarily necks and backs. Mechanical devices for separating tissue from the carcass were perfected and approved for use in establishments producing products under USDA inspection. Recently, mechanical separation has been extended to red meat. However, with mechanical separation, a quantity of small bone fragments also passes through the separation device. Tolerances of 1% bone in deboned poultry and 0.75% calcium in red meat were established.

A titrimetric calcium method (1) was modified and used in our laboratories to determine bone in poultry and was extended to red meat, but the method had never been collaboratively studied.

Collaborative Study

Six laboratories participated in a study on a poultry and beef hand-deboned samples, 2 mechanically separated poultry samples, 4 fortified poultry samples, and 4 fortified beef samples. No mechanically separated beef was being produced at the time of the study. Mechanically separated poultry Sample P_3 was fortified with 0.273 and 0.456% calcium chloride to provide Samples 4 and 5, respectively. The hand-deboned beef sample was fortified with 0.187, 0.562, 0.396, and 0.786% calcium.

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Calcium in Mechanically Separated Poultry and Beef

Titrimetric Method

Official First Action

24.D01 *Reagents*

(a) *Ethylenedinitrilotetraacetic acid dihydrate (EDTA).*—0.02M. Dissolve 7.44 g EDTA (99+% purity) in H_2O in 1 L vol. flask and dil. to vol. Stdze with ACS $CaCO_3$ primary std as in **36.050(d)** (3 significant figures).

(b) *Hydroxy naphthol blue indicator.*—Mallinckrodt Chemical Works.

(c) *KOH–KCN solution.*—Dissolve 280 g KOH in 500 mL H_2O. Cool, add 66 g KCN, dissolve, and dil. to 1 L in H_2O. *Caution:* Use care in handling of KCN. HCN is evolved on contact with acid or moisture. Flush titrd soln down drain in fume hood with ample *cold* H_2O.

(d) *Calcium carbonate.*—0.02M. Weigh 2.000 g ACS primary std $CaCO_3$ (dried 2 h at 100°) and transfer to 1 L vol. flask. Add 500 mL H_2O and 10–12 mL HCl (1 + 1). Heat just to boiling to dissolve $CaCO_3$. Cool and dil. to vol. with H_2O. Pipet 25 mL aliquot of EDTA (a) into 400 mL beaker and det. ratio of EDTA to $CaCO_3$ by titration as under *Determination* beginning ". . . add ca 50 mL H_2O, place on mag. stirrer. . ." Use av. of 3 detns.

24.D02 *Determination*

Weigh 10 g sample, transfer to 300 mL tall form beaker, add 30 mL HCl (1 + 1), several glass beads, cover with watch glass, and place on hot plate in fume hood. Bring to boil slowly, digest 20 min, cool, and filter (Whatman No. 4 paper) into 200 mL vol. flask. Wash filter paper with H_2O until 190 mL is obtained. Dil. to vol. and mix. Pipet 20 mL aliquot into 400 mL beaker, add ca 50 mL H_2O, place on mag. stirrer, and add

200–300 mg hydroxy naphthol blue indicator, stir, and adjust pH to 12.5 ± 0.2 with KOH–KCN soln. (Do not exceed pH 12.7, to prevent pptn. If pptn occurs, discard and repeat.) Add 10–25 mL 0.02M EDTA accurately measured to provide back-titrn of min. of 3 mL. Mix on mag. stirrer and titr. sample with 0.02M $CaCO_3$ to permanent purple end point. If back-titrn is <3 mL, repeat with larger aliquot of EDTA soln.

$$\% \text{ Calcium (Ca)} = (A - B \times R) \times M \times 4$$

$$\% \text{ Bone (for poultry)} = (\%\text{Ca} - 0.015)F$$

where A = mL EDTA added; B = mL $CaCO_3$ of back titrn; R = EDTA:$CaCO_3$ ratio; M = molarity of EDTA soln; 4 = meq. wt Ca (0.040 g) × 100(%); 0.015 = correction for natural Ca in poultry tissue; F = Ca-to-bone conversion factor (6.25 for broilers and fryers; 4.55 for turkeys and mature fowl).

Discussion

The raw data are listed in Table 1 for poultry and Table 2 for beef. Means and standard deviations, S_o for repeatability, S_L for laboratory bias, and S_x for reproducibility, are also listed. Data were submitted to the Youden (2) ranking test; Laboratory 4 was identified as an outlier. Data were re-examined excluding the results from Laboratory 4. Statistical data obtained by using the technique of Steiner (2) are also listed in Tables 1 and 2.

An examination of the data and the analytical method indicated that over the range of the study the data could be pooled to determine the standard deviations S_o, S_L and S_x. The data were adjusted and analyzed according to Steiner. Adjustment was made by subtracting the consensus mean value from the individual values to establish performance standards and to obtain an objective measure of the bias. Adjustment was performed on data from all laboratories. Mean differences and standard deviations were computed for each laboratory on each species, and for both species combined and are listed in Table 3. A bias of −0.03% calcium for Laboratory 4 was unacceptable. The use of the consensus mean for the adjustment for evaluation permits an objective measurement of bias which is not possible using the ranking technique of Youden. In addition, the statistical evaluation of each laboratory across all levels produces an estimate of laboratory repeatability, S_A. An investigation in the laboratory indicated that the standardization of solutions may have caused the bias.

The adjusted values were also submitted to a pooled analysis by Steiner's technique to obtain the statistical summary in Table 4. Tables 5 and

Table 1. Collaborative results for determination of calcium (%) in poultry

	Sample				
Lab.	P_1	P_2	P_3	P_4	P_5
1	0.024	0.112	0.288	0.552	0.712
	0.032	0.128	0.280	0.592	0.752
2	0.010	0.098	0.295	0.521	0.729
	0.032	0.092	0.279	0.530	0.744
3	0.056	0.104	0.264	0.608	0.728
	0.040	0.104	0.272	0.560	0.728
4	0.044	0.106	0.240	0.553	0.632
	0.008	0.080	0.256	0.516	0.664
5	0.066	0.118	0.318	0.563	0.722
	0.049	0.120	0.302	0.531	0.714
6	0.042	0.115	0.283	0.540	0.810
	0.060	0.112	0.281	0.568	0.738
All laboratories					
$\bar{X}$	0.0386	0.1074	0.2790	0.5528	0.7228
S_o	0.015	0.0090	0.0087	0.0244	0.0260
S_L	0.0111	0.0100	0.0197	0.0139	0.0370
S_x	0.0187	0.0135	0.0216	0.0281	0.0452
Excluding Lab. 4					
$\bar{X}$	0.0411	0.1103	0.2862	0.5565	0.7377
S_o	0.0119	0.0055	0.0080	0.0244	0.0266
S_L	0.0133	0.0099	0.0141	0.0146	0.0102
S_x	0.0178	0.0113	0.0162	0.0285	0.0284

Table 2. Collaborative results for determination of calcium (%) in beef

Lab.	Sample B_1	B_2	B_3	B_4	B_5
1	0.024	0.200	0.600	0.440	0.808
	0.032	0.232	0.600	0.488	0.872
2	0.040	0.207	0.576	0.414	0.838
	0.007	0.213	0.567	0.431	0.854
3	0.056	0.232	0.608	0.408	0.768
	0.048	0.232	0.568	0.424	0.792
4	0.004	0.180	0.540	0.424	0.746
	0.002	0.160	0.528	0.376	0.784
5	0.050	0.235	0.602	0.420	0.793
	0.049	0.239	0.601	0.444	0.761
6	0.036	0.231	0.596	0.431	0.839
	0.044	0.231	0.610	0.443	0.875
All laboratories					
$\bar{X}$	0.0327	0.216	0.583	0.4286	0.8108
S_o	0.0104	0.0111	0.0130	0.0221	0.0269
S_L	0.0169	0.0235	0.0252	0.0150	0.0363
S_x	0.0198	0.0260	0.0283	0.0267	0.0452
Excluding Lab. 4					
$\bar{X}$	0.0386	0.2252	0.5928	0.4343	0.8200
S_o	0.0113	0.0104	0.0137	0.0189	0.0269
S_L	0.0098	0.0089	0.0091	0.0128	0.0333
S_x	0.0150	0.0137	0.0165	0.0228	0.0428

Table 3. Analyst performance, bias, and repeatability

Lab.	Poultry $\bar{X}$	Poultry S_A[a]	Beef $\bar{X}$	Beef S_A	Combined $\bar{X}$	Combined S_A
1	0.00692	0.0176	0.01475	0.0268	0.01084	0.0224
2	−0.00728	0.0180	0.00048	0.0209	−0.0034	0.0194
3	0.00612	0.0194	−0.0062	0.0230	0.00275	0.0210
4	−0.0304	0.0292	−0.0398	0.0180	−0.0351	0.0241
5	0.01002	0.0174	0.00518	0.0235	0.0076	0.0203
6	0.0146	0.0272	0.01938	0.0178	0.01699	0.0225

[a] Standard deviation for laboratory repeatability.

Table 4. Statistical summary of pooled data (0.033–0.81% CA)

Statistic	Poultry	Beef	Combined
S_o	0.0174	0.0154	0.0165
S_{LS}	0.0125	0.152	0.0150
S_L	0.00717	0.0124	0.0072
S_x	0.0226	0.0250	0.0225

6 show results on analysis of variance of the data.

A recommendation from one collaborator to require a minimum back titration of 3 mL was incorporated into the method.

Recommendation

It is recommended that the method be adopted official first action for the determination of bone in poultry (in the absence of phosphates) and calcium in red meat.

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Randy Covington, Midwestern Laboratory, Science

Ned Deban, Western Laboratory, Science,

Table 5. Analysis of variance (poultry; acceptable laboratories)

Source of variation	SS	DF	MS	Variance ratio
Between labs	0.002676	4	0.00066913 (MS_L)	1.088* (MS_L/MS_{LS})
Between (adjusted) samples	0	4	—	—
Lab.-sample interaction	0.00982868	16	0.000614925 (MS_{LS})	2.01* (MS_{LS}/MS_o)
Between replicates	0.007615911	25	0.0003046364 (MS_o)	

* Not significant at 0.05 level.

Table 6. Analysis of variance (beef; acceptable laboratories)

Source of variation	SS	DF	MS	Variance ratio
Between labs	0.0070756	4	0.0017689 (MS_L)	2.52* (MS_L/MS_{LS})
Between (adjusted) samples	0	4	—	—
Lab.-sample interaction	0.01121775	16	0.00070119375 (MS_{LS})	2.94** (MS_{LS}/MS_o)
Between replicates	0.00595828	25	0.0002383312 (MS_o)	

* Not significant at 0.05 level.
** Significant at 0.05 level.

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Charles A. Scheib, Campbell Soup Co., Camden, NJ

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PESTICIDE FORMULATIONS

High Pressure Liquid Chromatographic Determination of Brodifacoum in Formulations: Collaborative Study

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A method is described for the determination of brodifacoum in technical and formulated material by high pressure liquid chromatography (HPLC). Samples of technical or formulation are dissolved in a solution of 1,3,5-triphenyl benzene in dichloromethane and methanol. The solution is injected into an HPLC system fitted with 25 cm Zorbax ODS column and an ultraviolet detector. The solution is eluted with a mixture of methanol, water, and acetic acid. Quantitative data are obtained by comparing peak areas of the compound and triphenyl benzene with those obtained by injecting a standard solution. Bait formulations are extracted into dichloromethane-formic acid, filtered, and concentrated by evaporation before chromatography. Nine collaborators made replicate determinations on 4 samples including technical material, a 0.1% powder, a 0.25% liquid, and a 0.005% pelleted bait formulation. They also carried out a recovery experiment on the bait product. Coefficient of variation (%) were 1.21 on the technical product, 13.49 on the powder, 2.40 on the liquid, and 11.06 on the bait. Insufficient data were generated to adequately quantitate the recovery factor. The method has been adopted official first action for the analysis of technical material, liquid, and bait formulations.

Brodifacoum, 3-[3-(4′-bromo[1,1′-biphenyl]-4-yl)-1,2,3,4-tetrahydro-1-naphthalenyl]-4-hydroxy-2*H*-1-benzopyran-2-one, is a new generation anticoagulant rodenticide active against a variety of rodent pests. It differs from conventional anticoagulant rodenticides in that the formulated product can kill rodents in a single feeding, it is effective against rodents resistant to conventional anticoagulants, and it presents a minimum hazard to humans and most domestic animals. It is the active ingredient of the products Talon, Havoc, and Volid (registered trademarks of ICI Americas Inc., Wilmington, DE) (1).

Methods of analysis for brodifacoum involving high pressure liquid chromatography (HPLC) are described in the literature (2–4). The principal product forms are baits containing 10–50 ppm active ingredient. Methods of analysis for bait formulations involve extraction, cleanup, concentration, and HPLC determination of the active ingredient. Yuen describes a method (2) which involves silica gel–Florisil column cleanup of a methanol–formic acid extract of the bait, evaporation of the eluate, and HPLC determination on the residue. Hall et al. describe a method (3) which involves fractionation of a methylene chloride–formic acid extract of bait on a preparative gel permeation column, evaporation, concentration, and HPLC determination. In tests, both methods gave less than 100% recovery of active ingredient from the bait. Yuen's method gave variable recoveries depending on the bait matrix used. The gel permeation technique gave consistent recoveries but we were unable to find sufficient laboratories equipped to carry out a collaborative study. Accordingly we investigated several high resolution HPLC columns to see if direct HPLC determination on bait extracts would give satisfactory recoveries and reproducibility. This collaborative study uses a 25 cm Zorbax ODS 5 μm column.

Collaborative Study

Nine collaborators analyzed 4 samples. These included one technical sample (No. 1), one powder sample (No. 2), one liquid sample (No. 3), and one bait sample (No. 4). In addition they were asked to carry out an extractability study which involved spiking blank pelleted bait (No. 4A) with a weighed amount of the liquid concentrate to simulate a 50 ppm bait and analyzing the product as bait. In addition to the samples,

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collaborators were supplied with analytical standard and triphenyl benzene.

Collaborators were requested to carry out trial injections of blank bait and bait material extracts to optimize separation on their particular instrument and column. They were requested to analyze each of the 4 samples and a spiked bait sample, using duplicate injections, in one day and to repeat the whole operation on a subsequent day. Collaborators were asked to submit all data and chromatograms to the Associate Referee.

Brodifacoum in Technical Material and Formulations

Liquid Chromatographic Method

Official First Action

6.D01 *Principle*

A weighed sample of tech. brodifacoum, brodifacoum conc., or bait ext is dissolved in triphenylbenzene internal std soln and detd by reverse phase liq. chromatgy and UV detection.

6.D02 *Apparatus*

(a) *Liquid chromatograph.*—Flow rate 1 mL/min; loop injection 10 μL; mobile solv. MeOH–H_2O–HOAc (94.2 + 5.0 + 0.8), filtered and degassed; UV detection at 254 nm with range to give peak hts ca 60–80% full scale. Retention times (min)—brodifacoum 6.2, internal std 11.7.

(b) *Column.*—25 cm × 4.6 mm Zorbax ODS 5 μm reverse phase column (DuPont Instruments Inc.).

(c) *Centrifuge.*—Equipped with 15 mL capped tubes.

(d) *Macerator.*—With 400 mL stainless steel cup/impeller assembly, such as Sorvall Omnimixer (DuPont Instruments Inc.).

(e) *Rotary evaporator.*—Fitted with vac. and cold H_2O supplies.

(f) *Filter.*—Spread 10 g Celite 545 on 9 cm No. 5 filter paper wetted with MeOH in buchner. Press filter and prewash with 30 mL MeOH.

6.D03 *Reagents*

(a) *1,3,5-Triphenylbenzene internal std soln.*—Accurately weigh ca 100 mg pure 1,3,5-triphenylbenzene into 500 mL vol. flask, dissolve in 200 mL CH_2Cl_2, and dil. to vol. with MeOH.

(b) *Brodifacoum std soln.*—Accurately weigh ca 100 mg brodifacoum std of known purity (ICI Americas Inc., PO Box 208, Goldsboro, NC 27530) into 100 mL vol. flask. Dissolve in 40 mL CH_2Cl_2. Dil. to vol. with MeOH. Transfer 10.0 mL each of brodifacoum std soln and internal std soln to 50 mL vol. flask and dil. to vol. with dilg soln.

(c) *Diluting soln.*—CH_2Cl_2–MeOH (2 + 3).

(d) *Extracting soln.*—CH_2Cl_2–formic acid (50 + 1).

Store reagents in tightly capped dark bottles to avoid evapn and decomposition. Check internal std soln for interfering components by injecting 10 μL into liq. chromatograph.

6.D04 *Determination*

(a) *Technical material.*—Accurately weigh ca 100 mg sample into 100 mL vol. flask. Dissolve in 40 mL CH_2Cl_2. Dil. to vol. with MeOH. Transfer 10.0 mL each of sample soln and internal std soln to 50 mL vol. flask. Dil. to vol. with dilg soln.

(b) *Powder concentrate.*—Accurately weigh amt sample contg ca 5 mg brodifacoum into 250 mL capped conical flask. Add 100 mL extg soln and shake 1 min. Filter thru Celite, using two 30 mL washes of extg soln. Evap. filtrate at 60° under vac. Dissolve residue in 20.0 mL dilg soln and 5.0 mL internal std soln.

(c) *Liquid concentrate.*—Accurately weigh amt sample contg ca 5 mg brodifacoum into 25 mL vol. flask. Add 5.0 mL internal std soln, and dil. to vol. with dilg soln.

(d) *Pelleted bait.*—Grind amt sample contg ca 2 mg brodifacoum in anal. mill. Transfer to tared macerator cup and accurately weigh. Add 250 mL extg soln and homogenize 10 min. Filter thru Celite using three 50 mL washings of extg soln. Rotary-evap. filtrate at 60° under vac. Dissolve residue in 8.0 mL dilg soln and 2 mL internal std soln. Centrf. sample to remove remaining solids.

Inject 2 or more aliquots of std soln into liq. chromatograph to set integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of std soln and 2 aliquots of sample soln in succession. Calc. response factor, R, for each:

$$R = \text{area brodifacoum peak} / \text{area internal std peak}$$

Peak hts can be used in place of peak areas for tech. material and bait formulations, but not for liq. formulations.

$$\text{Brodifacoum, \%} = (R/R') \times (W'/W) \times P \times F$$

where R and R' = av. response factor for sample and std solns, resp.; W and W' = mg sample and std, resp.; P = purity (%) of std; F = scaling factor = 1 for technical material, $1/20$ for powder and liq. concs, $1/50$ for pelleted bait.

Results and Discussion

All laboratories except 2 submitted both peak height and peak area data. The remaining 2 laboratories submitted peak height data only. The data from all laboratories were recalculated from raw data to check for arithmetical errors, to standardize on roundings and calibration techniques, and to generate individual injection analyses rather than means to allow for statistical manipulations by pairing. In certain cases, this has caused marginal variations between the data summary and results quoted by the collaborators.

An extractability factor was estimated as a percentage ratio of the analyses of the liquid concentrate spiked onto blank bait No. 4A and liquid concentrate No. 3. Because this factor was found to be heavily affected by problems in the analyses of Sample 3, an alternative extractability factor was estimated using the actual value for No. 3. Five of the laboratories used substitute columns in place of the one recommended. Two laboratories made substantial variations in rates of dilution and weights recommended. They were particularly concerned about damaging their columns with the concentrations suggested and preferred lower concentrations and a higher detector sensitivity. One laboratory reported results on 2 different columns. Variations are noted on the relevant tables.

Peak height and peak area data are reported in Tables 1 and 2. Comments from individual collaborators on problems they had experienced and a review of chromatograms for interference were used to reject results included in the boxed area in the tables from the statistical summary. One additional omission, based on a calculation for outliers, is the set of data from Collaborator 6 for peak height on Sample 3.

A summary of the statistics is included in Table 3. Precision within laboratories, s_r, is calculated from day to day pairs. In certain cases, omission of results has reduced the number below the level required to satisfy AOAC standards.

One oversight in our method development which appeared in the collaborative study was in the analysis of the liquid concentrate sample. Some collaborators had distorted active ingredient peaks. It is speculated that this is due to the brodifacoum initially chromatographing in the associated and the unassociated form even though we have acetic acid in the mobile phase. While peak area measurement by an electronic integrator can handle this, peak height measurement could give inaccurate results. This is not apparent in all the results and is presumably a function of injector design, volume, and concentration of the injection solution. This problem can be overcome by dilution of the sample (2 of our collaborators have data to demonstrate this), a smaller injection volume, or acid extraction of the sample which is formulated in a basic solubilizing agent. Because insufficient data were available from the collaborative study to modify the method, a caution will be included in the recommendations against the use of peak height measurements for liquid formulations. This was not a problem in the blank bait sample spiked with liquid concentrate because an acid extraction was included in the methodology.

An unaccountable level of variation was seen in the results for the powder sample (No. 2). This is an unacceptable level and it is not recommended that this technique be used for powder formulations until we have carried out further study.

A review of the data on the bait formulation analyses shows a higher level of variation than would be expected for a normal formulation method. However, this technique is being used for analysis of levels of active ingredient which would be considered residue levels if they occurred in crops and as such the level of variation is acceptable. It is interesting to note that despite the wide variety of columns used in this study there is reasonable agreement between bait analyses. Only Collaborator 5 had unacceptable chromatography on these samples. All the chromatograms showed a certain amount of low level interference. Only Collaborator 9 attempted quantitation of the interferences (see table notes).

A cleanup stage could be implemented in the method (3). However, this always involves a certain amount of lost sample, resulting in less than 100% recovery. It would appear from these results that the errors caused by interference are no more than errors typically experienced from cleanup losses. The results also suggest that there is no particular advantage in using peak height measurements rather than peak area

Table 1. Peak height data from collaborative study on brodifacoum

	Sample										Extractibility, %			
	1		2		3		4		4A		4A × 100 / No. 3		4A × 100 / 0.2612	
Collab.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1[a]	93.84	90.69	0.0960	0.0943	0.2234	0.2220	0.0046	0.0042	0.2906	0.2685				
	93.81	91.75	0.0962	0.0944	0.2266	0.2236	0.0046	0.0043	0.2906	0.2667	129.2	120.1	111.3	102.5
	93.82	91.22	0.0961	0.0943	0.2250	0.2228	0.0046	0.0043	0.2906	0.2676				
2[b]	90.55	92.14	0.1025	0.0890	0.2234	0.2128	0.0043	0.0040	0.2555	0.2289				
	90.02	92.18	0.1026	0.0885	0.2037	0.2313	0.0042	0.0040	0.2601	0.2304	120.7	103.5	98.7	87.9
	90.28	92.16	0.1025	0.0888	0.2136	0.2220	0.0043	0.0040	0.2578	0.2296				
3[c]	92.83	89.95	0.0757	0.0827	0.2561	0.2554	0.0030	0.0028	0.2474	0.2337				
	91.36	87.65	0.0749	0.0820	0.2650	0.2571	0.0030	0.0027	0.2457	0.2354	94.6	115.5	94.4	89.8
	92.09	88.80	0.0753	0.0823	0.2606	0.2563	0.0030	0.0027	0.2466	0.2346				
4[d]	88.64	90.63	0.0759	0.0791	0.2448	0.2404	0.0044	0.0041	0.2516	0.2374				
	88.26	91.18	0.0775	0.0790	0.2415	0.2443	0.0045	0.0041	0.2546	0.2321	104.1	96.8	96.9	89.9
	88.45	90.90	0.0767	0.0791	0.2432	0.2424	0.0044	0.0041	0.2531	0.2347				
5[e]	90.88	90.53	0.1005	0.0810	0.2123	0.2122	0.0064	0.0043	0.3029	0.2820				
	90.58	90.30	0.1004	0.0809	0.2128	0.2132	0.0066	0.0044	0.3018	0.2885	142.2	134.1	115.7	109.2
	90.73	90.42	0.1005	0.0810	0.2126	0.2127	0.0065	0.0044	0.3023	0.2853				
6	91.36	90.56	0.0678	0.0678	0.1779	0.2289	0.0043	0.0042	0.2844	0.2914				
	91.81	90.03	0.0681	0.0674	0.1795	0.2295	0.0043	0.0044	0.2908	0.2977	160.9	128.5	110.1	112.7
	91.59	90.29	0.0680	0.0676	0.1787	0.2292	0.0043	0.0043	0.2876	0.2945				
7[f]	89.80	92.29	0.0887	0.1061	0.2467	0.2683	0.0038	0.0040	0.2211	0.2237				
	89.69	92.54	0.0879	0.1061	0.2475	0.2672	0.0037	0.0039	0.2261	0.2240	90.5	83.6	85.6	85.7
	89.75	92.42	0.0883	0.1061	0.2471	0.2677	0.0037	0.0039	0.2236	0.2239				
8[g]	90.91	92.36	0.0876	0.0846	—	—	0.0043	0.0033	—	0.2390				
	87.61	93.21	0.0869	0.0839	—	—	0.0043	0.0034	—	0.2433	—	—	—	92.3
	89.26	92.79	0.0873	0.0843	—	—	0.0043	0.0033	—	0.2412				
9[h]	94.59	89.70	0.0921	0.0979	0.1589[j]	0.2138	0.0034	0.0045	0.2692	0.2709				
	93.29	90.76	0.0923	0.0925	0.1448[j]	0.2161	0.0034	0.0044	—	0.2697	177.3[j]	125.8	103.1	103.5
	93.94	90.23	0.0922	0.0977	0.1518[j]	0.2149	0.0034	0.0044	0.2692	0.2703				
9A[i]	93.23		0.0921		0.3096		0.0037		0.2754					
Alt. column	92.91		0.0909		0.3022		0.0037		0.2719		89.5		104.8	
	93.07		0.0915		0.3059		0.0037		0.2737					

[a] No peak area data reported. Did not quote column used.
[b] Ultrasphere ODS column substituted.
[c] Partisil PX5 10/25 ODS-2 column substituted. All samples diluted 25x.
[d] Partisil 10 ODS-2 30 cm column substituted.
[e] Lichrosorb RP-18 column substituted. Inadequate resolution Day 1, Samples 4 & 4A.
[f] Weight and dilution changes from those in method. Samples 4 & 5 filtered through paper, not Celite.
[g] No peak area data reported. Chromatography on 3 suspect; data not reported by collaborator.
[h] Peak heights not reported by collaborator. Measured and reported by Associate Referee. Problem with chromatography on Day 1, Sample 3. Collaborator estimated interference in 4 & 4A on Day 2 as 0.00008% and 0.0039%, respectively.
[i] Zorbax C8 25 cm column substituted. Collaborator estimated interference in 4 and 4A as 0.00065% and 0.033%, respectively. Peak heights not reported by collaborator. Measured and reported by Associate Referee.
[j] Chromatography/integration problem.

Table 2. Peak area data from collaborative study on brodifacoum

	Sample										Extractibility, %			
	1		2		3		4		4A		4A × 100 / No. 3		4A × 100 / 0.2612	
Collab.	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
1[a]	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2[b]	90.29	91.41	0.1026	0.0884	0.2527	0.2669	0.0041	0.0039	0.2267	0.2333				
	89.72	91.13	0.1021	0.0881	0.2552	0.2673	0.0039	0.0039	0.2291	0.2277	89.8	86.3	87.3	88.2
	90.01	91.27	0.1024	0.0883	0.2539	0.2671	0.0040	0.0039	0.2279	0.2305				
3[c]	94.23	97.59	—	0.0673	0.2497	0.2479	—	—	—	0.2567				
	—	92.40	—	0.0659	0.2598	0.1908	—	—	—	0.1912	—	102.1	—	85.8
	94.23	94.99	—	0.0666	0.2548	0.2193	—	—	—	0.2240				
4[d]	89.99	89.28	0.0813	0.0786	0.2623	0.2667	0.0050	0.0047	0.2745	0.2654				
	90.27	90.99	0.0786	0.0791	0.2618	0.2762	0.0051	0.0045	0.3130	0.2600	112.0	96.8	112.48	100.6
	90.13	90.13	0.0799	0.0789	0.2620	0.2714	0.0050	0.0046	0.2938	0.2627				
5[e]	88.90	91.17	0.0998	0.0814	0.2724	0.2640	0.0124[j]	0.0046	0.9089[j]	0.2996				
	88.85	90.40	0.0991	0.0811	0.2710	0.2664	0.0120[j]	0.0043	1.1285[j]	0.3761	374.9[j]	127.4	390.0	129.4
	88.87	90.79	0.0994	0.0812	0.2717	0.2652	0.0122	0.0045	1.0187	0.3379				
6	91.83	91.89	0.0691	0.0678	0.2732	0.2689	0.0039	0.0040	0.2515	0.2709				
	92.30	92.10	0.0693	0.0681	0.2748	0.2699	0.0039	0.0038	0.2599	0.2913	93.3	104.4	97.9	107.6
	92.06	92.00	0.0692	0.0679	0.2740	0.2694	0.0039	0.0039	0.2557	0.2811				
7[f]	92.24	89.92	0.0903	0.0993	0.2694	0.2608	0.0046	0.0043	0.2575	0.2421				
	92.30	91.02	0.0897	0.1041	0.2661	0.2602	0.0049	0.0041	0.3011	0.2409	104.3	92.7	106.9	92.5
	92.27	90.48	0.0900	0.1017	0.2678	0.2605	0.0047	0.0042	0.2793	0.2415				
8[g]	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9[h]	91.45	89.94	0.0907	0.0980	0.2073[j]	0.2467	0.0035	0.0042	0.2695	0.2687				
	94.27	90.64	0.0920	0.0984	0.2142[j]	0.2651	0.0035	0.0042	—	0.2586	127.9[j]	103.0	103.2	100.9
	92.86	90.29	0.0913	0.0982	0.2107[j]	0.2559	0.0035	0.0042	0.2695	0.2636				
9[i]	92.54		0.0895		0.2620		0.0036		0.2575					
Alt. column	87.33		0.0885		0.2647		0.0035		0.2477		95.9	—	96.7	—
	89.93		0.0890		0.2634		0.0035		0.2526					

[a–j] See Table 1.

Table 3. Statistical summary of results from AOAC collaborative analytical study on brodifacoum

Sample	1	2	3	4	4A	4A × 100 / No. 3	4A × 100 / 0.2612
				Peak Height			
N	36	36	24	32	24	12[b]	16[c]
$\bar{X}$	91.06	0.0870	0.2355	0.0040	0.2537	111.24	99.81
S_r	1.96	0.0074	0.0087	0.0004	0.0125	9.05	4.43
S_b	−1.02[a]	0.0083	0.0178	0.0004	0.0224	16.18	9.18
$\sqrt{S_r^2 + S_b^2}$	1.96	0.0111	0.0199	0.0005	0.0257	18.54	10.19
CV, %	2.15	12.73	8.43	13.75	10.12	16.66	10.21
				Peak Area			
N	22	24	20	20	16[c]	8[b]	10[b]
$\bar{X}$	90.79	0.0874	0.2663	0.0042	0.2591	97.45	99.76
S_r	0.98	0.0078	0.0064	0.0003	0.0226	7.91	6.70
S_b	0.50	0.0089	−0.0016[a]	0.0003	0.0136	3.62	5.05
$\sqrt{S_r^2 + S_b^2}$	1.10	0.0118	0.0064	0.0005	0.0263	8.70	8.39
CV, %	1.21	13.49	2.40	11.06	10.17	8.93	8.41

[a] Set $S_b = 0$.
[b] Too few data to publish summary statistics.
[c] Tentative summary statistics.

measurements to minimize errors caused by interference in the bait analyses.

The extractability factor estimates that the mean extractability is close to 100%. It is interesting to note that Sample 4 was formulated to give exactly 50 ppm brodifacoum. The mean analysis by a peak height measurement was 42 ppm (84% recovery) and by peak height measurement 40 ppm (80% recovery). This indicates that the spiking technique of incorporating liquid concentrate onto pelleted blank bait is not a true approximation of the real life situation where product is formulated and pelleted with the active ingredient in it.

Recommendations

It is recommended that the high pressure liquid chromatographic method for the determination of brodifacoum in technical, liquid, and bait materials be adopted official first action with the caution that peak area measurement is the preferred method of quantitation. The lowest concentration of bait material tested was 50 ppm. Further work should be carried out to investigate the applicability of the method to powder formulations, low concentration bait materials, and wax block materials. A modified method should be developed and collaborated for wax block bait formulations because these require substantial pretreatment to remove wax as an interfering component.

Test samples of formulation extracts without internal standard added should always be checked by users for interfering components.

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Gas-Liquid Chromatographic Determination of Endosulfan Technical and Formulations: Collaborative Study[1]

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Collaborating Laboratories: BASF AG (2 laboratories); Bayer AG; Biologische Budesanstalt; Boehringer Ingelheim; Hoechst AG (2 laboratories); J. Kepler Universitat (Analytische Chemie); Ligtermoet Chemie B.V.; Merck; Schering AG

The gas-liquid chromatographic determination of endosulfan technical and formulations was collaboratively studied with 11 laboratories. Fifteen samples were dissolved in toluene with di-(2-ethylhexyl)phthalate as the internal standard, chromatographed on a column of 10% OV-210, and detected by thermal conductivity. The average coefficients of variation were 0.55% for the 5 technical samples, 0.60% for the 5 wettable powders, and 0.68% for the 5 emulsifiable concentrates. The method has been adopted official first action.

Endosulfan (hexachlorohexahydromethano-2,4,3-benzodioxathiepin 3-oxide) is a mixture of 2 isomers, α-endosulfan and β-endosulfan. It is a pesticide that controls insects, bugs, foliar-feeding larvae, worms, and slugs on a wide variety of food, fiber, forest, tobacco, and ornamental crops. Infrared (1) and hydrolysis titration (2) methods have been reported, but they lack specificity. A gas-liquid chromatographic (GLC) procedure based on that reported by Zweig and Archer (3) is quick and precise and efficiently separates the active ingredient from interfering substances.

Collaborative Study

The method was tested in a collaborative trial by the Deutscher Arbeitskreis Für Pflanzenschutz-mittel Analytik (DAPA). Eleven laboratories were each sent one sample of technical endosulfan, one sample of 35% emulsifiable concentrate, and one sample of 50% wettable powder along with analytical standards of α-endosulfan and β-endosulfan, internal standard, and a copy of the method. Collaborators were instructed to analyze each sample 5 times for α- and β-endosulfan and report the average of 2 injections for each determination.

Endosulfan (Hexachlorohexahydromethano-2,4,3-benzodioxathiepin 3-Oxide) in Formulations

Gas-Liquid Chromatographic Method

Official First Action

CIPAC-AOAC Method

6.D16 ***Principle***

Sample is extd with toluene and α- and β-endosulfan isomers are detd sep. by thermal conductivity or flame ionization GLC, using di(2-ethylhexyl)phthalate as internal std.

6.D17 ***Apparatus***

(a) *Gas-liquid chromatograph.*—Suitable for on-column injection; equipped with thermal conductivity detector. Flame ionization detector may be used with proper diln of samples and stds.

(b) *Chromatographic column.*—3 mm id × 1.5 m (5 ft) glass column packed with 10% OV-210 on 80–100 mesh Chromosorb W-HP. Condition column ≥16 h, at 250°, using carrier gas at ca 25 mL/min. Operating conditions: injector 300°, detector 250°, column 230°, He carrier gas flow ca 60 mL/min.

6.D18 ***Reagents***

(a) *Toluene.*—GLC quality.

(b) *Internal std soln.*—15 mg/mL. Dissolve 15 g di(2-ethylhexyl)phthalate, 99%+, in toluene and dil. to 1 L with toluene.

(c) *Endosulfan std soln.*—Accurately weigh 0.300 g endosulfan of known α- and β-isomer content (Riedel de Haen Co., D-3016 Hanno-

[1] The method was accepted as a full CIPAC method (document 2901M) at the 24th meeting of the Collaborative International Pesticides Analytical Council 1980 (CIPAC).

[2] FMC Corp., 2501 Sunland Ave, Fresno, CA 93717.

[3] State Department of Agriculture, Salem, OR 97310.

The recommendation of the Associate Referee was approved by the General Referee and Committee A and was adopted by the Association. See the General Referee and Committee reports, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

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This report of the Associate Referee, J. E. Launer, was presented at the 96th Annual International Meeting of the AOAC, Oct. 25–28, 1982, at Washington, DC.

ver-Seelze 1, GFR) into 25 mL g-s flask. Pipet in 10 mL internal std soln, (**b**), mix, and store ≥20 min at 60° to ensure dissolution.

6.D19 *Preparation of Sample*

(**a**) *Technical endosulfan.*—Accurately weigh 0.300 g sample into 25 mL g-s flask, pipet in 10 mL internal std soln, (b), mix, and store ≥20 min at 60°.

(**b**) *Emulsifiable concentrates.*—Prep. as in (**a**), using wt equiv. to 0.300 g endosulfan.

(**c**) *Wettable powder.*—Accurately weigh sample contg 0.300 g endosulfan into 25 mL g-s flask, pipet in 10 mL internal std soln, (**b**) mix, store ≥20 min at 60°, and centrf.

6.D20 *Determination*

Inject 2 μL portions of std soln, (**c**), until response factor for each component varies <1% for successive injections. Inject duplicate 2 μL portions of sample soln followed by 2 μL portions of std soln. For each injection, calc. response ratio of α-isomer peak to internal std peak, and ratio of β-isomer peak to internal std peak, either as peak ht or digital integrator count for area. Retention time for each isomer and internal std should be the same for sample and std solns.

6.D21 *Calculation*

$$\% \ \alpha\text{-isomer} = C_\alpha = R_\alpha \times W' \times P_\alpha/(R'_\alpha \times W)$$

$$\% \ \beta\text{-isomer} = C_\beta = R_\beta \times W' \times P_\beta/(R'_\beta \times W)$$

where R_α and R'_α = response ratios of α-isomer peaks to internal std peaks for sample and std soln, resp.; R_β and R'_β = response ratios of β-isomer peaks to internal std peaks for sample and std soln, resp.; W and W' = wt (g) of sample and endosulfan std, resp.; P_α and P_β = % α- and β-isomers in std, resp.

Results and Recommendations

The relation between α- and β-endosulfan for all samples was nearly constant; thus, for the evaluation of data, the respective α- and β-endosulfan values were summed and one number for the sum is reported in Tables 1, 2, and 3. The results were evaluated statistically according to Massart et al. (4).

The original data allow a separate statistical evaluation of values for isomers. The ranges of within-laboratory standard deviations S_j and corresponding coefficients of variation are listed in Table 4. The ranges of mean values $\bar{X}_j$ of the individual laboratories are listed in Table 5. The results of the interlaboratory statistical evaluation are listed in Table 6.

The within-laboratory coefficients of variation (Table 4) range between 0.2% and 1.14%. The numbers are very similar for the different samples. Such values can be expected for trained laboratories. The mean values in Table 5 show relative differences in the range of 1.8–2.70%, which is considered satisfactory. From the reproducibility data (Table 6), critical differences between values of 2 laboratories calculated to about 2.3% (relative). The interlaboratory mean values $\bar{X}$ are in good agreement with the values derived from the wet chemical reference method (Table 6).

This statistical evaluation of the trial shows that the reproducibility is better than the comparability between different laboratories. This overall precision amounts to about 2.5% (relative) for all samples with a significance of 95%.

Reproducibility, r 95% = 2.83
× standard deviation of a single laboratory

Table 1. Collaborative results for determination of endosulfan technical (%)

Coll.	Value 1	Value 2	Value 3	Value 4	Value 5	$\bar{X}_j$	S_j	r_j (95)
1	97.38	96.95	96.60	96.76	97.39	97.01	0.36	1.15
2	93.6	94.3	94.3	99.2	—	—	—	—
3	97.33	96.56	97.39	97.88	97.22	97.28	0.47	1.86
4	94.98	94.45	96.39	94.38	95.15	95.06	0.84	3.29
5	95.01	97.75	96.56	96.92	95.98	96.44	1.03	4.03
6	97.4	97.2	97.1	98.2	97.4	97.46	0.43	1.69
7	97.46	97.52	97.08	97.60	97.20	97.37	0.22	0.86
8	96.32	95.93	96.14	95.74	95.94	96.01	0.22	0.86
9	96.77	96.63	96.31	97.31	96.34	96.67	0.41	1.59
10	96.42	96.43	96.00	96.01	96.72	96.32	0.31	1.45
11	96.29	96.69	95.53	96.32	95.49	96.06	0.53	2.08

Table 2. Collaborative results for determination of endosulfan WP (%)

Coll.	Value 1	Value 2	Value 3	Value 4	Value 5	$\bar{X}_j$	S_j	$r_j(95)$
1	49.80	49.91	50.33	50.04	50.03	50.02	0.198	1.57
2	50.5	51.0	51.0	47.4	—	49.98	1.73	7.79
3	50.96	50.87	51.01	50.89	50.71	50.89	0.114	0.45
4	49.95	50.04	48.75	49.31	49.83	49.58	0.541	2.12
5	50.17	48.78	49.45	49.61	49.71	49.54	0.504	1.98
6	50.4	50.7	50.7	50.3	50.8	50.58	0.22	0.86
7	50.56	50.33	50.52	50.41	50.36	50.44	0.10	0.39
8	49.90	50.09	50.14	50.30	50.19	50.12	0.15	0.59
9	50.29	50.22	50.52	50.28	50.08	50.28	0.16	0.58
10	50.27	50.73	50.68	50.58	50.40	50.53	0.19	0.75
11	49.62	50.38	50.13	50.41	50.64	50.24	0.39	1.53

Table 3. Collaborative results for determination of endosulfan EC (%)

Coll.	Value 1	Value 2	Value 3	Value 4	Value 5	$\bar{X}_j$	S_j	$r_j(95)$
1	34.39	34.18	34.54	34.33	33.58	34.20	0.372	1.67
2	35.4	35.4	35.6	36.9	—	35.83	0.732	3.25
3	34.16	33.73	34.08	33.83	34.22	34.00	0.213	0.84
4	—	33.41	33.36	33.07	33.09	33.23	0.177	0.80
5	34.19	34.57	34.40	33.53	34.23	34.18	0.395	1.55
6	34.6	34.6	34.3	34.3	34.2	34.40	0.187	0.73
7	34.06	34.26	34.08	33.77	34.01	34.04	0.18	0.71
8	33.82	34.01	34.12	33.76	33.94	33.93	0.14	0.55
9	34.54	34.36	34.78	34.15	34.11	34.39	0.279	1.10
10	33.73	33.81	33.79	33.91	33.71	33.79	0.08	0.31
11	33.85	33.45	33.84	34.16	34.08	33.88	0.28	1.1

Table 4. Range of within-laboratory standard deviations S_j and coefficients of variation

Sample	Range of S_j	Range of coefficients of variation, %
Endosulfan technical ($\alpha + \beta$)	0.22–0.84	0.23–0.87[a]
Thiodan WP 50 ($\alpha + \beta$)	0.10–0.50	0.20–1.00[b]
Thiodan EC 35 ($\alpha + \beta$)	0.08–0.40	0.23–1.14[a,c]

[a] One laboratory eliminated on basis of chi-square test.
[b] Two laboratories eliminated on basis of chi-square test.
[c] One laboratory eliminated because of outlying mean value.

Table 5. Range of the mean values $\bar{X}_j$

Sample	Range, %	Difference relative, %
Endosulfan technical ($\alpha + \beta$)	95.06–97.37	2.31[a]
Thiodan WP 50 ($\alpha + \beta$)	49.54–50.89	2.70[b]
Thiodan 35 EC ($\alpha + \beta$)	33.79–34.40	1.79[a,c]

[a] One laboratory eliminated on basis of chi-square test.
[b] Two laboratories eliminated on basis of chi-square test.
[c] One laboratory eliminated because of outlying mean value.

Table 6. Summary of statistical evaluation

Sample	Interlab. mean value, $\bar{X}$	Reproducibility, r (95)	Comparability, R (95)	Values with ref. method[a] (%)
Endosulfan technical ($\alpha + \beta$)	96.58	1.29	2.53	96.8
Thiodan WP 50 ($\alpha + \beta$)	50.29	0.73	1.27	50.2
Thiodan EC 35 ($\alpha + \beta$)	34.09	0.72	0.89	32.8

[a] Wet chemical method (2).

Table 7. Endosulfan 50 WP determined by CIPAC method using TCD gas chromatography and internal standard (%)

Sample	Injection 1	Injection 2	Injection 3	Mean	Range	Variance	SD ($n-1$)
7180	50.48	50.51	50.45	50.48	0.06	0.0009	0.03
7181	50.45	50.42	50.49	50.44	0.07	0.00045	0.02
7182	50.66	50.69	50.52	50.62	0.17	0.0082	0.09
7183	51.11	50.90	50.98	51.00	0.21	0.0112	0.106
7184	50.72	50.92	50.70	50.78	0.22	0.0148	0.12
7185	50.28	50.40	50.31	50.33	0.12	0.0039	0.06
7186	51.41	51.37	50.91	51.23	0.50	0.0772	0.28
7187	49.96	50.17	50.04	50.06	0.21	0.0112	0.106
7188	51.17	51.08	51.12	51.12	0.09	0.0020	0.045
7189	51.09	51.25	51.01	51.12	0.24	0.149	0.12

Table 8. Endosulfan 50 WP determined by FMC method using TCD gas chromatography and internal standard (%)

Sample	Injection 1	Injection 2	Injection 3	Mean	Range	Variance	SD ($n-1$)
7180	50.45	50.59	50.46	50.53	0.15	0.0054	0.07
7181	50.42	50.40	50.44	50.42	0.02	0.0004	0.02
7182	50.68	50.48	50.60	50.59	0.20	0.0101	0.10
7183	50.97	51.05	50.92	50.98	0.13	0.0043	0.066
7184	50.78	50.85	50.69	50.77	0.16	0.0064	0.08
7185	50.22	50.34	50.46	50.34	0.24	0.0144	0.12
7186	51.49	51.21	50.90	51.20	0.59	0.0871	0.30
7187	49.88	49.99	50.22	50.03	0.34	0.0301	0.17
7188	51.08	50.92	51.00	51.00	0.16	0.0064	0.08
7189	51.21	51.09	50.94	51.08	0.27	0.0183	0.135

Comparability, R 95 = 2.83
× standard deviation of the collaborative trial

A comparability of relative 2.5% can be considered good enough for a universal, simple method with high specificity. The within-laboratory reproducibility of about 1.5% (relative) is better than expected considering that 2 different elution peaks must be calculated and added.

Different types of gas chromatographic apparatus and detectors were used in the collaborative trial and gave consistent results. From this information, it can be deduced that flame ionization detectors with proper dilution of samples in standard can be used (3).

The Associate Referee compared this method with one used in his laboratory and, as indicated in Tables 7 and 8, found that the 2 methods agreed well. His company's method differs in that a 4 ft column with 16% SE-30 on 60–80 mesh Gas-Chrom Z is used with *n*-eicosane as an internal standard. Samples and standard are diluted to 10 mL with toluene–acetone (6 + 1). He recommends accepting the CIPAC method as tested, with the dilution changed to 10 mL toluene for samples and standard, and flame ionization detectors with sample and standard dilutions included as alternative detectors.

It is recommended that the GLC method for endosulfan as modified be adopted official first action.

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PESTICIDE RESIDUES

Multiresidue Method for Determination of Pesticides in Kiwifruit, Apples, and Berryfruits

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A rapid multiresidue procedure for fruits, based on methanol extraction, is presented. Water and highly water-soluble co-extractives are removed by partitioning the pesticides into toluene. Carbon-cellulose-Florisil cleanup is performed before gas chromatography on OV-225 with electron capture detection. A wide range of pesticides can be determined to 0.1 mg/kg without concentrating the extract. Gas chromatography of the crude toluene partition using SE-30 and an alkali flame ionization detector provides confirmatory data and allows detection of some carbamates. Dichlorvos, dimethoate, and acephate are determined directly in the methanol extract by flame photometric detection. High recoveries were obtained for 4 organochlorine insecticides, 13 organophosphorus insecticides, 2 synthetic pyrethroids, two *N*-methyl carbamates, and 10 fungicides. The method is economical of solvents, glassware, and time, and is recommended for routine surveillance of residue levels on fruit.

The development of the large apple and kiwifruit export industries in New Zealand has depended on growers' ability to produce fruit of very high quality which can meet the stringent quarantine standards of importing countries. This entails the use of comprehensive spray programs in the orchards with the concomitant risk of the harvested fruit having pesticide residue levels in excess of limits set by each country. To assist the horticultural industry with development of satisfactory spray programs and to monitor export orchard output, a convenient method was required for the analysis of a wide range of pesticide residues on fruit.

A number of multiresidue procedures have been developed which use extraction with a water-miscible solvent followed by partitioning with an organic solvent of limited water capacity. These methods ensure efficient extraction of residues from the sample, and do not require large amounts of drying agents. The wide range of polarities represented by modern pesticides means that partitioning systems must give good recoveries of polar as well as nonpolar compounds from aqueous based extracts. A number of solvent systems are satisfactory for multiresidue screening procedures; these include acetonitrile–dichloromethane (1, 2), acetonitrile–chloroform (3), acetone–dichloromethane (4–7), acetone–hexane or ethyl acetate (8), and dimethyl sulfoxide–petroleum ether (9). A multiresidue procedure for *N*-methyl carbamates uses methanol as the extractant followed by 2 partitioning stages before high pressure liquid chromatographic (HPLC) determination (10). All these methods require time-consuming evaporation of the partitioned solvent before further cleanup and analysis, either to concentrate the residues or to remove solvents that interfere with selective detectors.

Carbon adsorbent cleanup columns are used in a number of multiresidue analysis methods (1, 5, 6, 10, 11). The high capacity of carbon for plant pigments makes such columns particularly useful in the analysis of fruit, vegetable, and crop samples (12, 13). The carbon is generally mixed with an inert carrier such as cellulose powder or diatomaceous earth to improve the flow rate through columns.

Multiresidue methods which include organophosphorus compounds have generally made use of the very selective flame photometric or alkali flame ionization detectors. More recently, the availability of stable and sensitive conductimetric detectors with reactors selective to nitrogen, sulfur, or halogens has led to sophisticated screening systems based on a number of GLC column–detector combinations to provide complementary and confirmatory pesticide retention and response data (7). The high sensitivity and wide dynamic range of pulse-modulated electron capture detector designs (14, 15) allows the analysis of mixtures containing a range of pesticides, including those with only a moderate electron capture coefficient as well as highly electron-capturing polyhalogenated materials. It can thus function as a universal detector for pesticide residues provided sample

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extracts can be purified sufficiently to remove interferences.

Opportunities to make reductions in scale and simplifications in multiresidue procedures do not seem to have been investigated often. Friestad (9) has developed a rapid partitioning method using small solvent volumes. Micromethods have been reported for a number of organophosphorus insecticides (13, 16) and for a range of fungicides (17, 18).

In this paper we describe an economical multiresidue method suitable for fruits, based on sample extraction with methanol followed by toluene partitioning. Minicolumn cleanup of the toluene layer, using carbon-cellulose-Florisil, has been developed to provide purified extracts for primary screening of a wide range of pesticides by gas chromatography with electron capture detection.

METHOD

Apparatus and Reagents

(a) *Homogenizer.*—Waring blender with 1 qt glass jars.

(b) *Filter.*—9 cm diam., glass fiber, Whatman GFA.

(c) *Partition tubes.*—Boiling tubes, 100 mL, with B24 polyethylene stoppers.

(d) *Chromatography minicolumns.*—8 mm id × 12 cm long, with tapered tip.

(e) *Sample tubes.*—Calibrated centrifuge tubes, 15 mL, with Teflon-lined cap (Kimax 45166).

(f) *Gas chromatograph.*—Varian 3700, or equivalent, with pulse modulated ^{63}Ni electron capture detector (ECD) (280°C, amplifier range 10), and heated bead alkali flame ionization detector (AFID) (250°C, 10^{-11} A range). Nitrogen carrier gas flow 30 mL/min. Injector temperature 190°C. Column temperature 190°C, 9 min, then programmed 40°/min to 230°C.

(g) *GLC columns.*—1 m × 3 mm id glass packed with 5% OV-225 on 100–120 mesh Chromosorb W AWDCMS for ECD. 50 cm × 3 mm id glass packed with 10% SE 30 on 100–120 mesh Gas Chrom Q for AFID.

(h) *Recording integrator.*—Shimadzu CRIA.

(i) *Solvents.*—Toluene AR redistilled in glass, methanol AR (BDH or Ajax).

(j) *Adsorbents.*—Carbon-cellulose mixture: Mix 100 g acetone-washed cellulose powder (Whatman CF1) with 25 g acid-washed activated carbon (Nuchar C190N) as described by Brown (13). Florisil: Activate 24 h at 200°C, cool, add 4% water, mix thoroughly, and store in sealed jar.

Procedure

Take 100 g subsample of fruit. For large fruits (apples, kiwifruit) increased sensitivity may be obtained by analyzing peelings only from a larger subsample (200–300 g). Blend subsample with 80–110 mL methanol for 2 min. Filter extract through Buchner funnel and wash jar and cap with 30 mL methanol. Reblend filtercake with 100 mL methanol, and filter. Measure volume of combined filtrates. Place 20 mL aliquot in partition tube, add 10 mL toluene followed by 60 mL water containing 10% NaCl (w/v). Shake gently and let layers separate. Prepare chromatographic minicolumn: On top of glass wool plug add 0.5 g Florisil followed by 0.2 g carbon-cellulose mixture and 2 g anhydrous Na_2SO_4. Prewash column with 5 mL toluene. Add 5 mL of toluene layer from partition tube and collect eluant in sample tube. Elute with additional 5 mL toluene. Adjust total eluate volume to 10 mL. Make 3 μL injections into GC system of the eluate for ECD detection and of the crude toluene partition for AFID detection. Determine pesticide concentrations (ppm) in fractions based on area responses relative to standards. Calculate pesticide levels in sample using formula:

$$\text{Residue (mg/kg)} = (\text{ppm in fraction}/C) \times (\text{vol. methanolic extract (mL)}/\text{wt subsample (g)})$$

where C = concentration factor (1 for column eluant, 2 for toluene direct from partition, $10/V$ for column eluant concentrated or diluted to volume V).

Recovery Experiments

Subsamples of untreated fruit were fortified immediately before maceration by addition of 0.1 to 1 mg/kg of each pesticide in a small volume of acetone or toluene.

Completeness of extraction was checked by using field-treated apple or kiwifruit samples. Subsamples were put through the double methanol extraction procedure. The second filtercake was further blended with 100 mL toluene and filtered. The residues recovered in the methanol and toluene extracts were then determined. The effectiveness of a single methanolic extraction was determined on the same kiwifruit sample following the procedure of Krause (10). A 100 g subsample was blended for 5 min with 200 mL methanol and then filtered. A 20 mL aliquot of the filtrate was then carried through the toluene partition and cleanup procedure. These extraction efficiency tests were carried out in triplicate.

Occasionally, it may be necessary to concentrate extracts to obtain sufficient sensitivity in the GC step. The effect of evaporation on low level residues was checked as follows: A 20 mL aliquot of toluene containing 0.1 ppm each of diazinon, lindane, captan, iprodione, and azinphos-methyl was placed in a 125 mL round-bottom flask and concentrated to ca 0.5 mL on a rotary evaporator at a bath temperature of 55°C. Toluene washes were used to transfer the sample to a calibrated vial with a final volume of 2.0 mL before GC analysis.

Results and Discussion

Recoveries for 31 pesticides carried through the method are given in Table 1. Most recovery results are for kiwifruit but some were carried out with apples, boysenberries, or tamarillos.

Table 1 also lists relative retention times on OV-225 and approximate relative responses to the electron capture detector, using chlorpyrifos as the standard (19). RRT values beyond 5.0 are not isothermal due to the temperature program used to speed up elution of later-eluting components. Therefore, they cannot be used for comparative purposes but serve to give the elution order.

Completeness of extraction of field-incurred residues was checked by reblending samples with toluene following the methanol extraction. The residues extracted are listed in Table 2 and show that the double methanol blending recovered greater than 90% of the total residues of phosmet, azinphos-methyl, and chlorpyrifos extracted from kiwifruit or apples. The single methanol blending was not as effective, recovering only 77–85% of the residues from kiwifruit. Wheeler et al. (20, 21) have shown that a single methanol homogenization is effective in extracting carbamate residues from plant material. The higher water solubility of carbamates and the use of the more efficient Polytron mechanical-ultrasonic blender probably account for their high recoveries.

A double blending is used in this method to ensure effective extraction of nonpolar pesticides. The first methanol blend removes most of the water from the sample, allowing the second blend to better dissolve lipophyllic material as was indicated by the virtually complete extraction of plant pigments. The volume of methanol used in blending is kept to the minimum necessary for effective homogenization to avoid excessive dilution of the residues.

Some pesticides are unstable in aqueous methanol particularly in the presence of acidic co-extractives of fruit. For example, a methanol extract of kiwifruit containing phosmet and carbaryl showed a 7% and 12% decrease in the respective residues on storage for 24 h at −4°C. Unless the residues of interest can be shown to be stable, it is advisable to partition the methanolic extract within 1 h of preparation. Losses of the pesticides listed in Table 1 are negligible under these conditions as evidenced by the high recoveries achieved for most compounds (Table 1). Apart from this minor disadvantage, methanol is an inexpensive and effective solvent for pesticide residue extraction. We have also generally found the AR grade of sufficient purity

Table 1. Relative retentions, relative responses, and recoveries for pesticides

Pesticide	RRT[a]	Rel. response[b]	Recovery (%)[c]
Dichlorvos	0.17	1.2	17
Diazinon	0.41	0.01	94
Etrimfos	0.54	0.09	94
Lindane	0.69	4.1	105
Chlorpyrifos	1.00	1.0	94[d]
Vinclozolin	1.19	2.2	93
Dicofol	1.28	0.2	85
Carbaryl	1.36	0.01	88[d]
Endosulfan	1.37	1.8	86
Malathion	1.50	0.12	108
Acephate	1.5	0.02	0[e]
Chlorfenvinphos	1.60, 1.93	0.3	85
Dichlofluanid	1.61	1.5	85
Dimethoate	1.63	0.25	24[f]
Methiocarb	1.72	0.01	87
Prothiofos	1.76	1.3	92
Triadimefon	1.98	0.80	95[f]
Dieldrin	1.84	2.0	101
Pirimiphos-methyl	1.92	0.01	104
Parathion	1.93	0.62	103[d]
Folpet	2.84	0.64	88[e]
Captan	3.32	0.68	85
DDT	3.61	1.1	97
Endosulfan B	3.94	1.8	94
Bupimirate	4.04	0.40	104[d]
Propargite	4.47	0.02	88
Dinocap	5.75, 5.9, 6.2	1.0[g]	81[f]
Permethrin	5.8, 6.2	0.36[g]	79
Captafol	6.12	0.36	90
Iprodione	6.52	0.60	97
Phosmet	7.03	0.66	98
Azinphos-methyl	8.13	0.16	94
Cypermethrin	8.7, 9.6	0.30[g]	92

[a] Retention time relative to chlorpyrifos on 5% OV-225, 190°C for 9 min, then 40°/min to 230°C. Chlorpyrifos retention time: 1.82 min.

[b] Area response to electron capture detector relative to chlorpyrifos. Chlorpyrifos response 1×10^6 counts/ng.

[c] From untreated fruit samples fortified at 0.1–1 mg/kg level. Recoveries are from kiwifruit except where noted.

[d] From apples.

[e] From boysenberries.

[f] From tamarillos.

[g] Sum of multiple peaks.

to use without redistillation. These are considerable advantages given the large volumes of solvent consumed in routine pesticide residue analysis.

The carbon-cellulose-Florisil minicolumn provided sufficient cleanup for ECD analysis of fruit extracts to below 0.1 mg/kg for most pesticides in Table 1. The quantity of carbon-cellulose is chosen to be not much in excess of that required to hold back plant pigments. Use of larger quantities of carbon can result in losses of some pesticides such as pirimiphos-methyl and azinphos-methyl. Although Nuchar C-190N is no longer available, Nuchar S-N is reported to be a satisfactory substitute (10). The small plug of Florisil beneath the charcoal is required to reduce interferences in the ECD chromatograms and also to prevent a fall-off of GC response following injection of extracts.

Figure 1 shows a chromatogram for a number of pesticides on OV-225. This packing provides good resolution of the pesticides tested including pairs such as captan/folpet and iprodione/phosmet that are difficult to separate on less polar phases. The jump in ECD baseline with the fast temperature program was acceptable and decreased with column use. A more gradual program was inconvenient in that the slowly rising baseline gave frequent false triggering of the integrator. The low responses of diazinon and pirimiphos-methyl to the ECD (Table 1) do not compromise detection limits because their short elution times result in sharp peaks. Achieving low detection limits with this method without concentrating the extract requires chromatography of sub-nanogram quantities of pesticide. OV-225 packing has proved to be the most suitable in this respect of a number of column packings tested. Provided the column was carefully conditioned at 250°C and then stabilized with a number of injections of extracts and standards, reproducible calibrations were obtained. A number of pesticides were noted to be susceptible to column activity. In particular, captan suffered a rapid fall-off in response following injections of extracts that still contained chlorophyll pigments.

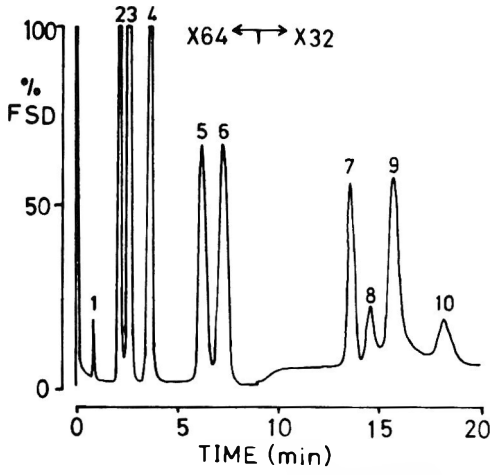

Figure 1. ECD chromatogram of 10 pesticides at the 2 ng level. Conditions: column 1 m × 3 mm id, 5% OV-225, 190°C 9 min, 40°/min to 230°C. ECD range 10. 1, diazinon; 2, chlorpyrifos; 3, vinclozolin; 4, dichlofluanid; 5, folpet; 6, captan; 7, captafol; 8, iprodione; 9, phosmet; 10, azinphos-methyl.

Figures 2 and 3 show ECD chromatograms of untreated and treated kiwifruit and apple samples taken through the method. The virtual absence of interferences allows detection limits of below 0.1 mg/kg to be achieved for most pesticides listed in Table 1. The blanks for boysenberries and a number of other berry fruits are similarly clean. The additional peaks marked A and B (Figure 2) from the extract of field-treated kiwifruit are *N*-(mercaptomethyl)-phthalimide and devinylated vinclozolin, which are breakdown products of phosmet and vin-

Table 2. Extractability of residues (mg/kg) from field-treated fruit

	Kiwifruit		Apples	
Extraction	Phosmet	Azinphos-methyl	Chlorpyrifos	Azinphos-methyl
Double methanol[a]	7.8 ± 0.2	0.51 ± 0.05	0.43 ± 0.03	0.73 ± 0.11
Toluene	0.3 ± 0.1	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
Total	8.1	0.53	0.46	0.78
Single methanol[b]	6.9 ± 0.3	0.41 ± 0.06	—	—

[a] 100 g fruit was blended with 100 mL methanol, filtered, and the cake was re-extracted with a further 100 mL methanol and filtrates were combined. The cake was then re-extracted a third time with 100 mL toluene.
[b] 100 g fruit was blended with 200 mL methanol, and filtered.

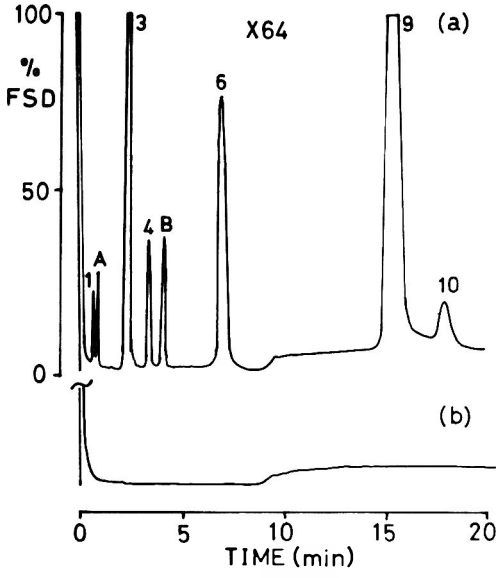

Figure 2. ECD chromatograms of kiwifruit extracts. See Figure 1 for conditions and peak identification. (a) Field-treated fruit; (b) untreated fruit.

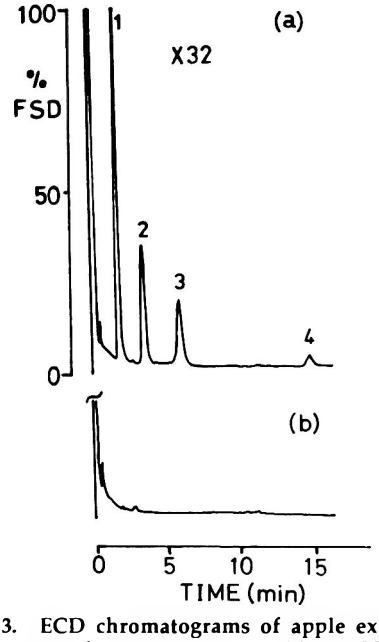

Figure 3. ECD chromatograms of apple extracts. Conditions: column 1 m × 3 mm id, 5% OV-225, 200°C 9 min, 40°/min to 230°C. ECD range 10. (a) Untreated fruit fortified with 0.2 mg/kg each of 1, chlorpyrifos; 2, parathion; 3, captan; 4, azinphos-methyl. (b) Untreated fruit.

clozolin, respectively, formed on the fruit before harvest (22).

Recoveries for bupimirate, carbaryl, dimethoate, and methiocarb (Table 1) were determined

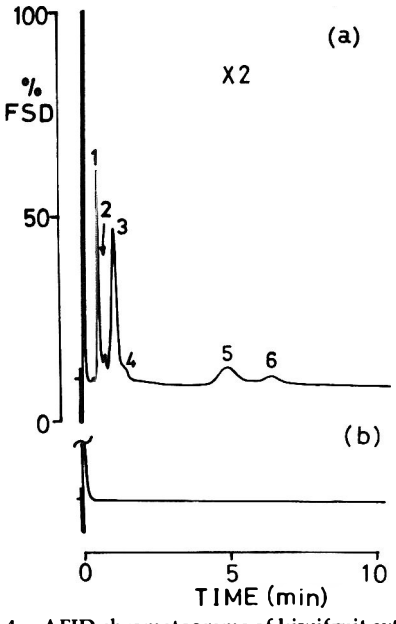

Figure 4. AFID chromatograms of kiwifruit extracts. Conditions: column 0.5 m × 3 mm id, 10% SE-30, 190°C. AFID range 10^{-11} A. (a) Untreated fruit fortified with 0.2 mg/kg each of 1, diazinon; 2, vinclozolin; 3, chlorpyrifos; 4, captan; 5, phosmet; 6, azinphos-methyl. (b) Untreated fruit.

in the upper layer of the partition using the SE-30 column and AFID detection. These pesticides are either not fully recovered from the cleanup column or do not chromatograph well on OV-225. The selectivity of the AFID is adequate to provide interference-free analysis of these materials in the toluene-based extracts of fruit, without column cleanup. However, frequent repacking of the head of the GLC column is required to preserve the response of the labile carbamates.

The response of a kiwifruit extract containing 0.2 mg/kg each of several pesticides and that of an untreated control are shown in Figure 4. The SE-30/AFID column-detector combination provides confirmation for the presence of a number of pesticides detected with the ECD. In particular, the enhanced relative response of the organophosphorus compounds to the AFID is useful. The greater range of pesticides detected with ECD and the higher separating power and resolution of the OV-225 column make that combination the most suitable for primary screening.

The poor recoveries of dichlorvos, dimethoate, and acephate by this method (Table 1) are due to inefficient partitioning of these very polar materials into toluene from aqueous methanol. The

flame photometric detector (P mode) has been found to be sufficiently selective to allow direct analysis of these pesticides in the crude extracts. The methanolic extracts of kiwifruit or tamarillos are partitioned with hexane to remove nonpolar co-extractives. Good sensitivity and recoveries have been achieved at the 0.1 mg/kg level. However, standards must be made up in control extract to avoid high apparent recoveries, and column life is limited. Similar findings have been reported by Luke et al. (7) using direct injection of acetone extracts. The poor response of propargite to the ECD (Table 1) requires that the flame photometric detector (S mode) be used for low level residues (less than 1 mg/kg).

If residue levels are very low the toluene extract can be concentrated by rotary evaporation. The high boiling point of toluene requires a bath temperature of 55°C for a reasonable evaporation rate and this may cause loss or degradation of some pesticides. However, recoveries for a range of pesticides concentrated 10-fold from 0.1 ppm in toluene were greater than 90% including relatively volatile diazinon and lindane.

When analyzing large fruits we have found it convenient to extract only the peelings which gives a 3-5-fold effective concentration without the need for the cumbersome evaporation step. Analysis of field-treated apple, kiwifruit, and tamarillo samples containing residues of a number of pesticides showed that for residue levels of 0.5 mg/kg and above (whole fruit), over 95% of the residue was in the skin.

The proportion of residue in the flesh may reach 10-20% of lower level residues where fruit has been treated with materials having systemic action or where frequent spraying was used early in the growing season. However, skin sampling gives reliable detection of samples containing residues exceeding set maximum residue limits (MRL) on a whole fruit basis or residues of pesticides that are unregistered or have no set MRL. Thus for enforcement purposes, skin analyses are adequate and flesh analyses should only be necessary where accurate data on actual dietary intake of pesticide residues is required.

This method has been applied to the analysis of a large number of fruit samples. In addition to kiwifruit, apples, and boysenberries, the following crops have been studied without serious interference or recovery problems: black currants, blueberries, feijoas, raspberries, and tamarillos. Over a 2 year period, several hundred kiwifruit samples from grower orchards have been analyzed. The mean difference between residue levels on duplicate fruit samples averaged over all samples and all pesticides detected above 0.2 mg/kg was 15%. This figure includes variability due to sampling and indicates a good overall reproducibility for the method.

The method is relatively rapid and economical. A trained technician can analyze 12 samples per day including washing glassware. The range of fruits and pesticides with which the method has been tested suggests that its applicability could be broadened to include other pesticides and crops.

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Simple, Sensitive Technique for Detection and Separation of Halogenated Synthetic Pyrethroids by Thin Layer Chromatography

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Thin layer chromatography employing silver nitrate-impregnated alumina G plates has been extended for the routine detection and confirmation of the identity of halogenated synthetic pyrethroid insecticides. Of the solvent systems tried, maximum resolution was achieved by using *n*-hexane-benzene (45 + 55) and *n*-hexane-chloroform (60 + 40), which gave distinct separations of the *cis*- and *trans*-isomers of permethrin and cypermethrin from fenvalerate and decamethrin. Possible interference of HCH isomers and DDT and its analogs can also be checked by a concurrent run of the sample mixture in these 2 systems. A minimum of 50 ng each of *cis*- and *trans*-permethrin and cypermethrin isomers and fenvalerate and decamethrin can be positively detected. Using this technique, the minimum detectable limit of permethrin was 0.1 ppm in the cleaned up extracts of tomato plant and fruit and soil samples.

Residue analysis of halogenated synthetic pyrethroid insecticides such as permethrin, cypermethrin, decamethrin, and fenvalerate has been mainly carried out by gas-liquid chromatography (GLC) using an electron capture detector (ECD) which permits their estimation at picogram levels (1). Because of the lack of specificity of the EC detector, the identity of these insecticides has been established by additional GLC analysis using columns with stationary phases of differing polarity (2, 3), by derivatization of the compounds (4), and by mass spectroscopy (5). Characterization of the nature of the residues of these insecticides has also been made by autoradiographic thin layer chromatography (TLC), using ^{14}C-labeled compounds, especially in metabolism studies, where the unlabeled compounds were detected by visualization on silica gel 60 F254 chromatoplates under UV light (5–11). Chromogenic reagents, viz., phosphomolybdic acid (20% w/v in ethanol) for the detection of permethrin, cypermethrin, and decamethrin (11) and palladium chloride (0.5% w/v in 12N HCl) for the detection of decamethrin (10), have also been used. Recently *cis*- and *trans*-permethrin were resolved in a hexane-acetone-benzene (9 + 1 + 1) solvent system and detected either by visualizing silica gel 60 F254 plates under UV light or by using phosphomolybdic acid reagent (12). Crop surface residues of allethrin, permethrin, pthalthrin, and resmethrin were separated by successive developments with benzene-carbon tetrachloride (1 + 1) and hexane-ether-formic acid (70 + 30 + 1). Hexane extracts, cleaned up by hexane-methyl cyanide partitioning, were used for spotting, and compounds were quantitated by dual wavelength (230 and 370 nm) densitometry (13). The present paper describes the use of silver nitrate, a more sensitive and hitherto not reported chromogenic reagent, for the detection of halogenated pyrethroids. Their comparative resolution in different solvent systems in the presence of isomers of HCH and DDT and related compounds is also presented.

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METHOD

Apparatus and Reagents

(a) *Thin layer chromatography.*—20 × 20 cm glass plates; slurry applicator (Perfit, Ambala, India); development tank (Kontes); UV lamp, 120 watts, without filter (M/s Toshniwal Instruments, Madras, India).

(b) *Solvents.*—Acetone, benzene, chloroform, ethanol, ether, ethyl acetate, *n*-hexane, HCl (analytical grade).

(c) *Other materials.*—Aluminum oxide 60 G neutral (Merck, Type E); silica gel G (BDH); $AgNO_3$ (BDH); phosphomolybdic acid (Merck); palladium chloride (BDH); anhydrous Na_2SO_4 (BDH); NaCl (BDH); acidic aluminum oxide (BDH).

(d) *Pesticide reference standards.*—Permethrin (*cis:trans* 36.6%; 55.3% purity, ICI, United Kingdom); *cis*-permethrin (99.6% purity, ICI); *trans*-permethrin (99.8% purity, ICI). Cypermethrin (94.2% purity), decamethrin (90% purity), fenvalerate (99.0% purity), HCH (alpha-, beta-, gamma-, delta-isomers), *p,p'*-DDE, *p,p'*-DDD, *o,p'*-DDT, and *o,p'*-DDT (95% purity) were obtained from U.S. Environmental Protection Agency, Research Triangle Park, NC.

Extraction and Cleanup of Samples for Permethrin Residues

Extract 50 g substrate (tomato fruits/leaves) with 3 portions (100, 50, 50 mL) of *n*-hexane-

Table 1. Mean R_f values (× 100) of halogenated synthetic pyrethroids, HCH-isomers, DDT, and related compounds

Solvent system	Permethrin		Cypermethrin		Deca-methrin	Fenvalerate	HCH				DDT			
	cis	*trans*	*cis*	*trans*			δ	β	γ	α	*p,p'*-DDD	*p,p'*-DDT	*o,p'*-DDT	*p,p'*-DDE
n-Hexane	8	4	0	0	0	0	3	6	18	28	18	43	56	65
Benzene	88	85	83	80	82	80		81, 86[a]				90[a]		
Chloroform	90	88	80[b]		80	80		88, 90[a]				92[a]		
Hexane–benzene														
80 + 20	34	24	15		10	8	24	30	42	51	54	60	63	69
70 + 30	44	31	20	18	19	16	32	38	51	58	60	70	72	76
60 + 40	66	56	40	36	38	33	41	50	75	79		92, 94[a]		
55 + 45	75	64	52	44	46	40	46	62	78	94		90, 84[a]		
50 + 50	82	75	58	52	55	47	52	66	79	89		92, 96[a]		
45 + 55	86	79	64	56	60	52	48	68	80	90		92, 96[a]		
40 + 60	88	86	78		78	76	52	74	86	92		96, 98[a]		
Hexane–$CHCl_3$														
80 + 20	51	40	31	26	28	25	24	34	46	57	61	66	68	73
70 + 30	79	69	46	37	44	36	36	44	54	60	64	76	79	84
60 + 40	87	82	57	49	53	45	40	63	73	88		89, 91[a]		
50 + 50	90	84	61	58	60	55	48	66	76	93		91, 93[a]		
40 + 60	92	88	74		72	67	60	70	82	88		94, 97[a]		
Hexane–$CHCl_3$–benzene														
45 + 5 + 50	75	67	48	42	45	39	44	65	79	86		92, 96[a]		
40 + 10 + 50	84	77	59	61	60	55	49	68	82	88		94, 96[a]		

[a] Incomplete resolution.
[b] No separation.

Table 2. Comparative resolution ($R_f \times 100$) of synthetic pyrethroids, HCH isomers, DDT, and analogs by 2 solvent systems

	Hexane–benzene (45 + 55)		Hexane–chloroform (60 + 40)	
Compound	Mean	SD	Mean	SD
cis-Permethrin	86	1.5	87	0.6
trans-Permethrin	79	0.6	82	2.1
cis-Cypermethrin	64	1.2	57	2.1
trans-Cypermethrin	56	1.2	49	0.6
Decamethrin	60	1.5	53	1.2
Fenvalerate	52	2.0	45	2.0
delta-HCH	48	1.7	40	2.1
beta-HCH	68	2.1	63	2.1
gamma-HCH	80	0.6	73	2.6
alpha-HCH	90	1.0	88	0.6
DDT group	92	1.2	89	1.0
	96	2.1	91	2.1

acetone (9 + 1) by blending 2 min in presence of 50 g granular anhydrous Na_2SO_4. Filter macerate through glass wool plug into 500 mL separatory funnel. Wash extract with three 100 mL portions of 5% aqueous NaCl, discarding lower aqueous phase and any emulsified materials each time. Pass hexane extract through anhydrous Na_2SO_4 before concentrating to 2–3 mL in rotary vacuum evaporator for further cleanup. For soil, extract 50 g after adding 25 mL water by the same procedure but without addition of Na_2SO_4. After partitioning, use the concentrated hexane fraction directly for TLC.

Column Cleanup

Fit small glass wool plug in neck of a 15 × 40 cm glass column to support a 1 cm layer of anhydrous Na_2SO_4, 40 g activated (1 h at 110°C) acidic aluminum oxide, and 2 cm layer of Na_2SO_4. After prewashing the adsorbent with 100 mL *n*-hexane, transfer concentrated extract to column. Discard first fraction of 100 mL *n*-hexane and elute permethrin with *n*-hexane–ethyl acetate (95 + 5). Concentrate and use for TLC.

Preparation of TLC Plates

Prepare slurry of 40 g alumina G in 50 mL 0.1% $AgNO_3$ in water (80 mL for 40 g silica gel G) for 5 plates and coat plates with uniform 250 μm layer with applicator. Air-dry plates before activating 1 h at 110°C. Spot standard solutions of insecticides and test compounds 2 cm apart, 3 cm above base line. Use hot air dryer to prevent spreading of spots during application. Place plates in developing tank presaturated with solvents for 3 h. Let compounds elute up to 15 cm. Remove plates and air dry. Expose plates under UV lamp at distance of 10–15 cm for 10–20 min.

Results and Discussion

All the halogenated synthetic pyrethroids appeared as black spots on a white background as are obtained with organochlorine insecticides, using the $AgNO_3$–UV detection system. Because plates coated with alumina G gave better contrast with the background than those coated with silica gel G, further resolution studies were carried out with alumina G stationary phase, using different systems of single solvents with varying polarities and different solvent combinations. The 4 insecticides are very close in polarity, with log-octanol-water partition coefficient values of 6–7 (14), therefore, the R_f values are close in most of the solvent systems (Table 1). Because the spots were generally elliptical, with diameters not exceeding 5 mm vertically and up to 8 mm horizontally, the compounds did not overlap in the solvent systems *n*-hexane–benzene (45 + 55) and *n*-hexane–chloroform (60 + 40), which resolved the geometric isomers of permethrin and cypermethrin from fenvalerate and decamethrin. The mean R_f values (× 100) and their standard deviations (Table 2) clearly indicate that concurrent runs of the compounds in these 2 systems can check the interference of HCH isomers and DDT and its analogs. This is significant because these ubiquitous organochlorine compounds could interfere in the $AgNO_3$–UV detection system. No further improvement in resolution could be achieved by using combinations of even 3 solvents.

By this technique, a minimum of 50 ng each of *cis*- and *trans*-permethrin, *cis*- and *trans*-cypermethrin, fenvalerate, and decamethrin could be

positively detected. However, the earlier reported chromogenic reagents phosphomolybdic acid and palladium chloride were 20 and 40 times less sensitive (minimum detectable amounts 1000 and 2000 ng, respectively, for all the compounds). This technique has been employed in this laboratory for confirming the identity of permethrin residues in tomato plant and fruit and in soil samples. Using fortified samples, the minimum detectable limit was 0.1 ppm by spotting 100 μL (1 g sample equivalent) of the cleaned up extracts.

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Gas-Liquid Chromatographic Screening Method for Six Synthetic Pyrethroid Insecticides

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A method was developed for simultaneously determining 6 synthetic pyrethroid insecticides (fenpropanate, tetramethrin, permethrin, cypermethrin, fenvalerate, and deltamethrin) in apple, orange, grape, lemon, tomato, green pepper, and paprika, and sugar beet root and leaves. The pesticides were extracted with acetone and partitioned into methylene chloride. The extract was purified by column chromatography on active carbon-magnesia-diatomaceous earth and then on alumina. Analysis by gas-liquid chromatography with ^{63}Ni electron capture detection was completed in 6 min on a 3% SE-30 liquid phase, using a short column. Recoveries from fortified samples ranged from 68 to 89% in the range of 0.3-1.0 ppm and from 67 to 83% in the range of 0.02-0.2 ppm.

The rapid increase in the application of synthetic pyrethroid insecticides requires a method by which as many pyrethroids as possible can be screened in various plant samples. The present method was tested for simultaneous determination of 6 pyrethroids: fenpropanate (S 3206, SD 41706); tetramethrin (Neo-Pynamin, Phthalthrin, FMC 2960, S 1103, Pyrotox); permethrin (Permethrine, Ambush, NRDC 143, ICI PP557, WL 43479, FMC 33297, S 3151, Pounce); cypermethrin (Ripcord, WL 43467, NRDC 149, PP 383, Cymbush); fenvalerate (Sumicidin, Pydrin, S 5602, WL 43775); deltamethrin (decamethrin, Decis, FMS 45498, NRDC 161, RU 22974, Supermethrin). The 2-step adsorption chromatographic cleanup presented here enables the residue determination with limits of determination in the range of 0.005–0.05 ppm, depending on the detectability of pesticides and the chromatographic interferences of plant extracts. We tried to find gas-liquid chromatographic (GLC) conditions where the stereoisomers of the individual pesticides appear in a single peak while different compounds are resolved from each other. The cleaned up extract may contain several other types of pesticides, including chlorinated hydrocarbons, which can be analyzed applying suitable packings (1). An additional objective was to minimize the time required for GLC analysis of pyrethroids to produce a rapid screening procedure for many samples. A search of the literature failed to reveal any published, rapid method for simultaneous analysis of synthetic pyrethroid pesticides from various plant samples (2–8), although cypermethrin, fenvalerate, and permethrin were recovered by the multiresidue method of Ambrus et al. (9). Their extraction and cleanup procedures were used in this paper.

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METHOD

Chemicals

(a) *Pesticides.*—Fenpropanate [(±)-alpha-cyano-3-phenoxybenzyl-(±) -2,2,3,3-tetramethylcyclopropanecarboxylate] (99.5%) and fenvalerate [(±)-alpha-cyano-3-phenoxybenzyl-(±)-2-(4-chlorophenyl)-3-methylbutyrate] (98.5%) were supplied by Sumitomo Chemical Co. Ltd, Osaka, Japan. Cypermethrin [(±)-alpha-cyano-3-phenoxybenzyl-(±) -*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] (97%) was provided by Shell Research Ltd, Sittingbourne Research Centre, Sittingbourne, Kent, UK. Permethrin [(3-phenoxybenzyl-(±) -*cis,trans*-3-)2,2-dichlorovinyl-2,2-dimethylcyclopropanecarboxylate] (98.5%) and tetramethrin [(1,3,4,5,6,7-hexahydro-1,3-dioxo-2*H*-izoindol-2-yl) -methyl-2,2-dimethyl-3-(2-methyl-1-propenyl) cyclopropanecarboxylate] (98%) were supplied by Chinoin, Budapest, Hungary. Deltamethrin [(±)-alpha-cyano-3-phenoxybenzyl-(±)-*cis,trans*-3- (2,2-dibromovinyl)-2,2- dimethylcyclopropanecarboxylate] (98%) was obtained from Roussel, Uclaf, France.

(b) *Active carbon.*—Powder, pure (Reanal, Budapest, Hungary; or Fluka AG, 9470 Buchs, Switzerland) was washed with hot 1M HCl, refluxed 4 h, washed to neutral with water, and placed in an oven at 95–100°C to constant weight.

(c) *Magnesia.*—Analytical grade (Reanal) 400 g MgO was washed with absolute ethanol, filtered, dried, and heated 4 h at 140°C.

(d) *Diatomaceous earth.*—Acid-washed by the manufacturer (Reanal; or Celite 545 AW from Supelco, Inc., Bellefonte, PA 16823), heated at 400°C for 8 h.

(e) *Aluminum oxide.*—Woelm 200 neutral, activity grade V, 19% water added (ICN Pharmaceuticals GmbH. Co. 3440 Eschwege, GFR).

All solvents were redistilled in all-glass apparatus and checked by gas chromatography.

Apparatus

(a) *Gas chromatograph.*—Packard Becker Typ. 419, equipped with constant current, linearized ^{63}Ni electron capture detector. Operating conditions: temperatures (°C)—injector 240, column 230, detector 270; nitrogen carrier gas 17.5, and 18 mL/min purge.

(b) *GLC columns.*—Column I.—50 cm long, 1.75 mm id U-shape Pyrex glass column filled with 3% SE-30 on 100–120 mesh Gas-Chrom Q. *Column II.*—90 cm long, 2 mm id coiled glass column filled with 1.5% SP-2250 + 1.95% SP-2401 on 100–120 mesh Supelcoport (Supelco, Inc., Supelco Park, Bellefonte, PA 16823).

(c) *Chromatographic columns.*—With glass stopcocks. *Column I.*—1.8 cm id for the (1 + 2 + 4) mixture of active carbon, magnesia, and diatomaceous earth. *Column II.*—1.0 cm id for alumina.

Extraction

Weigh 50 g analytical sample and blend 3 min with 150 mL acetone, using high-speed homogenizer (e.g., Ultra-Turrax, Janke and Kunkel KG, IKA Werk). Filter macerate with suction through Buchner funnel. Rinse head of generator and jar, and wash filtrate with 25 mL acetone.

Transfer extract to 1 L separatory funnel, add 450 mL water containing 4% sodium sulfate, and mix. Extract with 100, 50, and 50 mL portions of methylene chloride, shaking separatory funnel vigorously for 3 min at each step, and filter extracts through 30 g anhydrous Na_2SO_4. Rinse Na_2SO_4 layer with 20 mL methylene chloride. Evaporate combined methylene chloride extracts to dryness with vacuum rotary evaporator at 35°C and dissolve extract in 5 mL benzene.

Column Chromatographic Cleanup

(a) *Mixed column.*—Weigh 7 g (1 + 2 + 4) mixture of active carbon, magnesia, and diatomaceous earth, suspend in 40 mL benzene, and transfer by portions to 1.8 cm id chromatographic column plugged with glass wool. Place 1 cm sea sand at top of column. Let solution pass through column, wash adsorbent with 40 mL methylene chloride, and let liquid elute to upper surface of adsorbent bed. Pipet equivalent of 10 g aliquot of sample in 1 mL benzene onto column and elute pesticides with 150 mL methylene chloride. Remove methylene chloride from eluate, using rotary evaporator at 35°C, and dissolve extract in 2 mL benzene.

(b) *aluminum oxide column.*—Place 8 g neutral aluminum oxide W 200, activity grade V, into 1 cm id column above glass wool plug. Use gentle vibration during filling. Wet adsorbent with 10 mL *n*-hexane. Pipet half the cleaned extract obtained from the mixed column onto the aluminum oxide bed in 1 mL benzene solution. Elute pesticides with 30 mL *n*-hexane (fraction 1). This fraction contains fenpropanate, permethrin, cypermethrin, fenvalerate, and deltamethrin. Elute tetramethrin with 30 mL *n*-hexane–ethyl ether (70 + 30) (fraction 2). Concentrate the 2 fractions separately to desired volume (usually 5 mL). This concentrate is suitable for analysis by electron capture gas-liquid chromatography.

Gas-Liquid Chromatography

Pesticides were analyzed under GLC conditions given above. Detector responses were linear for synthetic pyrethroids examined in the range of 0.005–0.5 ng. The calibration lines of each standard passed through the origin. Relative retention times were based on retention time of chlorpyrifos. *p,p'*-DDT was selected as a reference substance for chlorinated pesticides. Measurement was based on peak height, compared with a standard of appropriate concentration. The maximum loading of gas chromatographic system is 1–3 mg equivalent to cleaned crop sample.

Results and Discussion

Recoveries from fortified apple, grape, orange, lemon, tomato, whole (green) pepper, and paprika, and sugar beet root and leaf samples are given in Table 1. Not all insecticides were tested in each plant (all were tested in apple, orange, grape, and lemon) but from the column chromatographic features and from low levels of gas chromatographic interferences, one may presume the applicability of the method for all mentioned pesticides and samples. Recoveries and limits of determination are given in Table 1. At least 5 measurements were carried out at each level.

In Figures 1 and 2, typical chromatograms of standards and fortified and control samples are shown for comparison at 2 fortification levels. The concentration of active ingredients in the standard solution and in the final volume of fortified sample extract had been selected to be

Table 1. Recovery of insecticides from crops, with different column chromatographic cleanup, and limit of determination

Pesticide	Fortification level, ppm	Recoveries, %			Limit of detn, ppm
		Mixed adsorbent only	Mixed + N Al_2O_3		
			Fraction 1	Fraction 2	
Fenpropanate	0.1	90–95	76–85	—	0.007–0.01
	0.02		77–83	—	
Permethrin	0.5–0.8	81–93	83–89	—	0.02–0.03
	0.1		79–83	—	
Cypermethrin	0.3–1.0	83–93	71–81	—	0.01–0.02
	0.05		78–82	—	
Fenvalerate	0.3–0.8	80–86	77–81	—	0.005–0.01
	0.05		67–71	—	
Deltamethrin	0.3–1.0	80–88	68–76	—	0.005–0.01
	0.05		67–69	—	
Tetramethrin	0.5	64–75	—	62–71	0.02–0.05

the same; therefore peak heights on both chromatograms should be the same for complete recovery.

The limit of determination changed slightly from sample type to sample type. Some co-extractants from plant materials interfered more or less with fenpropanate, permethrin, and cypermethrin, as in the apple sample, for example, as shown in Figure 2A. As a result of slightly different chromatographic interferences arising from different sample types, a range is given for the limit of determination in Table 1.

For rapid screening of many different samples, the one-step extraction seems to be effective.

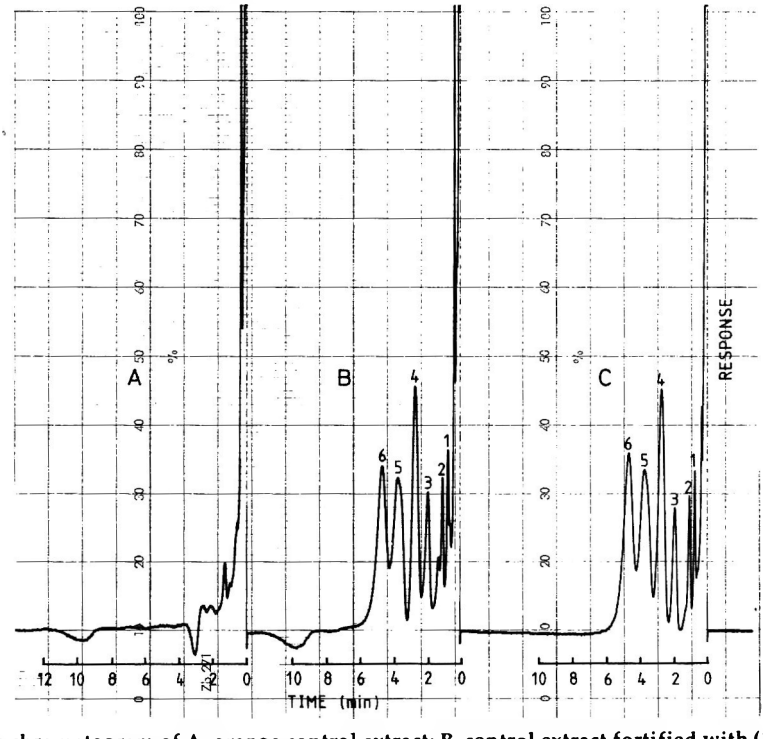

Figure 1. Gas chromatogram of A, orange control extract; B, control extract fortified with (1) *p,p'*-DDT at 0.025 ppm, (2) fenpropanate at 0.1 ppm, (3) permethrin at 0.5 ppm, (4) cypermethrin at 0.25 ppm, (5) fenvalerate at 0.25 ppm, (6) deltamethrin at 0.25 ppm; C, standard mixture with (1) 0.075 ng (2) 0.03 ng (3) 0.15 ng, (4) 0.075 ng, (5) 0.075 ng, (6) 0.075 ng, respectively. 3% SE-30 on 100–120 mesh Gas-Chrom Q; 0.5 m long, 1.75 mm id column at 230°C. Injected amount: 0.3 mg extract cleaned on mixed and Al_2O_3 columns.

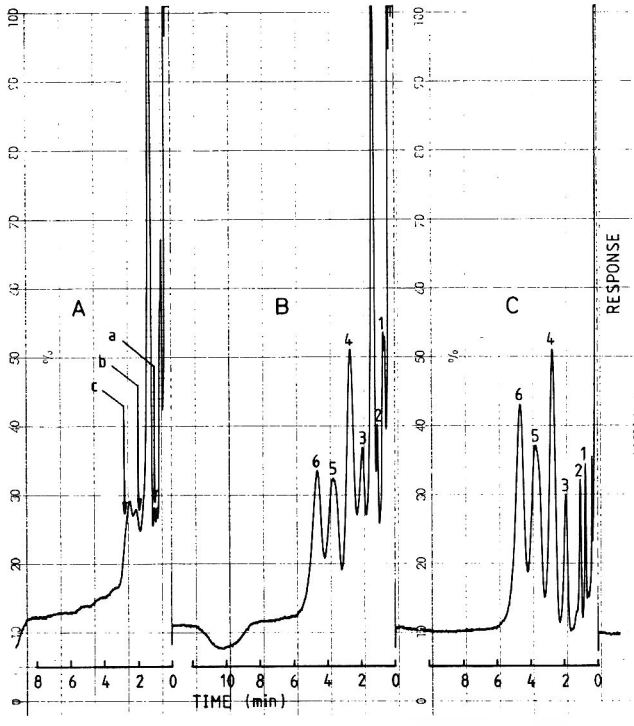

Figure 2. Gas chromatogram at low level of A, apple control extract; B, control extract fortified with (1) *p,p'*-DDT at 0.005 ppm, (2) fenpropanate at 0.02 ppm, (3) permethrin at 0.1 ppm, (4) cypermethrin at 0.05 ppm, (5) fenvalerate at 0.05 ppm, (6) deltamethrin at 0.05 ppm; C, standard mixture with (1) 0.0075 ng, (2) 0.03 ng, (3) 0.15 ng (4) 0.075 ng, (5) 0.075 ng, (6) 0.075 ng, respectively. 3% SE-30 on 100-120 mesh Gas-Chrom Q; 0.5 m long, 1.75 mm id column at 230°C. Injected amount: 1.5 mg equivalent to sample extract, cleaned on mixed and alumina columns. The arrows a, b, c show interferences of fenpropanate, permethrin, and cypermethrin, respectively, from plant materials.

Table 2. Relative retention time of insecticides on 2 liquid phases

Pesticide	Retention time		
	Relative to chlorpyrifos[a]	Relative to chlorpyrifos and aldrin[b]	
Aldrin	—	0.77	1.00 (60 s)[c]
Chlorpyrifos	1.00 (22 s)[c]	1.00 (78 s)[c]	1.30
p,p'-DDT	2.43	3.03	3.94
Tetramethrin	3.24	6.91	8.98
Fenpropanate	3.41	5.98	7.76
Permethrin	6.03	10.71	13.92
		11.85	15.40
Cypermethrin	8.51	19.46	25.30
		21.34	27.74
Fenvalerate	11.5	29.29	38.08
		33.06	42.98
Deltamethrin	14.59	40.31	52.40
		44.71	58.12

[a] 3% SE-30 on 100–120 mesh Gas-Chrom Q, 0.5 m × 1.75 mm column, 230°C, injector 240°C.
[b] 1.5% SP-2250 + 1.95% SP-2401 on 100–120 mesh Supelcoport, 0.9 m × 2 mm column, 200°C, injector 220°C.
[c] Absolute retention time.

Extraction efficiency was tested with field-treated apple samples; filter cake re-extraction, repeated twice, gave only a 9% additional recovery of cypermethrin, which is in good agreement with the experiences of Chapman and Harris (7).

When higher residue levels (0.2 mg/kg) are suspected (e.g., samples from supervised trials),

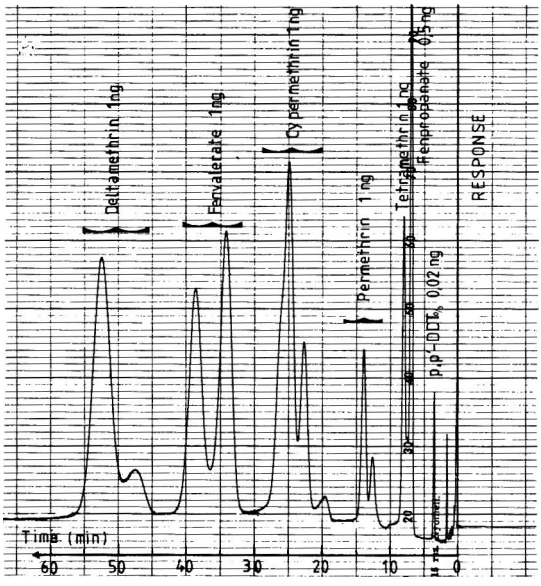

Figure 3. Gas chromatogram of some insecticides on 1.5% SP-2250 + 1.95% SP-2401 phase coated on 100-120 mesh Supelcoport, 0.9 m long, 2 mm id column at 200°C; injector 220°C.

one-step cleanup will suffice (either mixed column with 10 g sample loading or neutral aluminum oxide column with 5 g sample loading). Then the final extract is diluted and the chromatographic system is loaded with 0.2–0.3 mg sample only.

Since no resolution or only partial resolution of isomers occurs on apolar liquid phases (e.g., dimethyl-polysiloxane), which facilitates the quantitation of the residues, the SE-30 phase is recommended for rapid screening and quantitation. Retention times, relative to chlorpyrifos and aldrin, are summarized in Table 2. Experiments were done to optimize measurements on more polar, e.g., OV-17, QF-1, OV-225, liquid phases but neither the sensitivity, resolution, nor analysis time was sufficient. Nevertheless, these phases can be used to confirm individual components, but with the possible resolution of diastereomer isomers complicating the quantitation.

The stereo isomers diastereomers are partially resolved on polar packing, depending on the polarity, as shown, e.g., on 1.5% SP-2250 + 1.95 % SP-2401 mixed phase, which is widely used in gas-liquid chromatographic analysis of chlorinated pesticides (see Figure 3). In addition, there is a great difference in analysis times and sensitivities on packings of different polarity. The apolar phase such as SE-30, is the best from both aspects. Regulatory work requires the determination of the sum of the isomers; therefore, the quantitation is always carried out on an apolar phase. The characteristic pattern of the partially resolved isomers on more polar, e.g., on phenyl, trifluoropropyl, and cyano silicone phases, is useful for confirmation and, thus, the relative response factors and identification of individual isomers were not studied.

On the basis of structural similarities, one can assume that this method can be extended for the residue analysis of other pyrethroid insecticides. This method is useful not only in screening samples containing synthetic pyrethroids but the same cleanup extract will also contain the common chlorinated pesticides. Those can be analyzed from the final extract.

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Applicability of a Multiresidue Method and High Performance Liquid Chromatography for Determining Quinomethionate in Apples and Oranges

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The AOAC multiresidue method for nonpolar pesticide residues in nonfatty foods has been coupled with a high performance liquid chromatographic (HPLC)-fluorometric system for determining quinomethionate residues on apples and oranges. Quinomethionate is extracted with acetonitrile, and coextractives are removed with liquid-liquid partitioning and Florisil adsorbent using the AOAC multipesticide residue method for nonfatty foods. The quinomethionate fraction is then chromatographed on an HPLC octyl-bonded column and detected in-line with a fluorescence detector using 362 nm excitation and 395 nm emission. Recovery studies were conducted with apples fortified with quinomethionate at 0.05 ppm and oranges at 0.05 and 0.5 ppm. The recoveries averaged 100% (range 92-108) at the 0.05 ppm fortification level and 102% (range 93-110) at the 0.5 ppm level.

The multiresidue methodology concept of pesticide analysis, which is based on analysis of a food commodity for a variety of pesticides by a single method that is capable of simultaneously determining the individual residues, was developed in the 1950s. Application of this concept led to the development of the well established AOAC multiresidue method (1) for nonpolar organohalogen and organophosphorus pesticides, which uses gas-liquid chromatography (GLC) for determining the individual pesticides. This multiresidue methodology approach has been adopted in the development of a method for the *N*-methylcarbamate insecticides using a high performance liquid chromatographic (HPLC) determinative technique (2). Recently the extraction and coextractive removal steps of the carbamate method were shown to be applicable to several naturally fluorescent pesticides which are determined by HPLC and several organophosphorus pesticides which are determined by GLC (3). As part of this laboratory's ongoing efforts to increase the applicability of multiresidue methods to additional pesticides, the highly fluorescent pesticide quinomethionate, also known as chinomethionat (BSI and ISO common names, respectively), was studied.

Quinomethionate (6-methyl-1,3-dithiolo-[4,5-*b*]quinoxalin-2-one) is an acaricide and fungicide which has been commercially available for use on crops since the 1960s. Numerous countries permit its use on fruits and vegetables; however, its principal use is on fruits (4–6). The tolerance for quinomethionate on crops in the United States (7) ranges from 0.5 ppm on oranges to 0.05 ppm on apples and pears. Various analytical techniques have been published for determining quinomethionate residues in crops. Colorimetry (8), thin layer chromatography (9, 10), and GLC (11) have been used in single residue methods for determining quinomethionate. In the multiresidue method of Luke et al. (12), quinomethionate was determined by using a gas chromatograph equipped with a flame photometric detector operated in the sulfur mode. Recently, Argauer (13) reported the HPLC of quinomethionate on a cyano-bonded column, with fluorescence detection of the eluted pesticide.

The purpose of the work reported here was to investigate the applicability to quinomethionate of the extraction and cleanup procedures of the AOAC multiresidue method for organochlorine and organophosphorus pesticides in nonfatty foods (1), and the use of a high performance liquid chromatograph with a fluorescence detector for the determination of quinomethionate.

METHOD

Principle

Quinomethionate is extracted from crops with acetonitrile in a Polytron homogenizer. Crop coextractives are removed by diluting the filtered extract with water and partitioning quinomethionate into petroleum ether. Chromatography on a Florisil column removes remaining coextractives by eluting the quinomethionate with mixed solvent systems consisting of hexane–methylene chloride–acetonitrile. The collected eluate is evaporated to dryness and the residue is dissolved in acetonitrile. The quinomethionate is chromatographed on an HPLC

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octyl-bonded column by using an acetonitrile–water mobile phase and is detected in-line with a fluorescence detector by using 362 nm excitation and 395 nm emission.

The AOAC general method for organochlorine and organophosphorus pesticides (1) was used with the following additions/changes:

General Reagents

Sec. **29.002** (1) plus the following Florisil eluant mixtures (14):

(a) *Eluant 1.*—20% methylene chloride in hexane. Dilute 200 mL methylene chloride to 1 L with hexane.

(b) *Eluant 2.*—50% methylene chloride–0.35% acetonitrile–49.65% hexane. Pipet 3.5 mL acetonitrile into 500 mL methylene chloride and dilute to 1 L with hexane.

(c) *Eluant 3.*—50% methylene chloride–1.5% acetonitrile–48.5% hexane. Pipet 15 mL acetonitrile into 500 mL methylene chloride and dilute to 1 L with hexane.

General Apparatus

Sec. **29.005** (1) plus the following:

(a) *Vacuum rotary evaporator.*—Model RE Rotavapor (Brinkmann Instruments, Inc., Westbury, NY 11590). Maintain solution in condensing coils and around receiving flask at −15°C. (Refrigerated water–antifreeze solution works well.) Use vacuum pump fitted with manometer and needle valve to control vacuum in evaporator.

(b) *Swinny filter holder.*—13 mm filter size (No. XX3001200, Millipore Corp., Bedford, MA 01790).

(c) *Miltex filters.*—5 μm, 13 mm diameter, white, plain (No. LSWP 01300, Millipore Corp.).

HPLC Reagents

(a) *HPLC acetonitrile.*—UV grade distilled-in-glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Before use, degas acetonitrile in glass bottles by applying vacuum and slowly stirring solvent with magnetic stirrer 5 min.

(b) *Ultrapure water.*—Prepare by using Milli-Q water purification system (Millipore Corp.). Degas water as described above for HPLC acetonitrile.

(c) *Pesticide HPLC standard solutions.*—Dissolve pesticide standard(s) (EPA/FDA Reference Standards, Environmental Protection Agency, Research Triangle Park, NC 27711) in HPLC grade acetonitrile to give 1 μg/mL concentration or as needed. Store solution(s) in actinic glassware, and when not in use store in refrigerator. Standards stored in this manner are stable for several months.

HPLC Apparatus

See ref. 2.

Operating parameters.—Adjust mobile phase flow rate to 1.50 ± 0.02 mL/min at 50% acetonitrile in water. Equilibrate system at 12% acetonitrile in water for 10 min, inject sample, and begin 30 min linear gradient to 70% acetonitrile in water, and then 100% acetonitrile for additional 5 min. Operate column oven at 35°C. Operate carbamate hydrolysis chamber at ambient temperature when determining quinomethionate. Set fluorescence detector excitation and emission wavelengths at 278 and 306 nm, respectively, for establishing detector sensitivity with carbofuran as reference. Set detector photomultiplier gain to low and time constant to 1 s. Adjust sensitivity so that 50 ng carbofuran produces 50% full-scale response on printer plotter (recorder) at attenuation of 5. For determination of quinomethionate use 362 nm excitation and 395 nm emission.

Extraction

See sec. **29.011(b)** (1).

Coextractive Removal

Partitioning.—See sec. **29.011(e),(f)** (1).

Chromatography.—Sec. **29.033** modified as follows: Add weight of activated Florisil determined from lauric acid absorption value (sec. **29.002(i)**) to 22 mm id chromatographic tube (sec. **29.005(b)**). Gently tap chromatographic column to settle Florisil. Top column with ca 12 mm anhydrous granular Na_2SO_4. Wet column with 40–50 mL hexane. Use 250 mL ₹ 24/40 round-bottom flasks to collect eluates. Transfer petroleum ether or hexane solution of sample extract to column, letting it pass through at ca 5 mL/min. Rinse container (and Na_2SO_4 if present) with two ca 5 mL portions of hexane, transfer rinsings to column, and rinse walls of chromatographic tube with additional small portions of hexane. Elute column at ca 5 mL/min with 200 mL eluant 1. Change receivers and elute with 200 mL eluant 2. Change receivers and elute with 200 mL eluant 3. (Note: Quinomethionate may be eluted by eluant 3 as well as eluant 2. Analyst should check elution pattern of quinomethionate with Florisil lot being used.) Place 250 mL ₹ 24/40 trap on round-

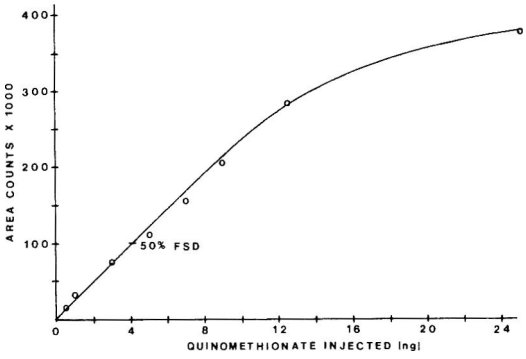

Figure 1. Quinomethionate response data.

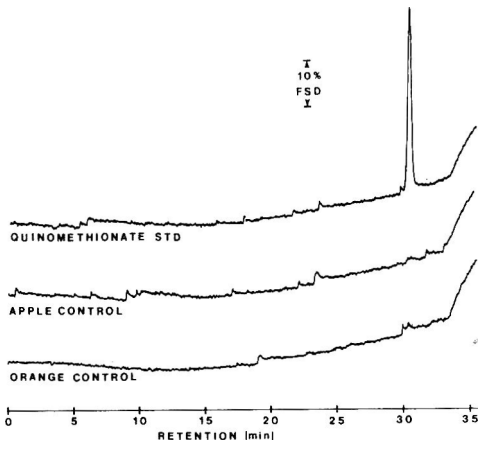

Figure 2. HPLC chromatograms of crop controls (200 mg) and quinomethionate standard (5 ng).

bottom flask containing eluate 2 and attach to vacuum rotary evaporator. Apply vacuum slowly to minimize frothing. After full vacuum is applied, slowly place round-bottom flask in 35°C water bath and evaporate solution just to dryness. Immediately pipet 5 mL HPLC acetonitrile into 500 mL round-bottom flask to dissolve residue. Pour acetonitrile sample solution into 10 mL glass syringe containing Swinny filter holder with 5 μm filter. Push acetonitrile solution through filter with syringe plunger, collecting filtrate in 10 mL centrifuge tube or other suitable container. Repeat for eluate 3.

Note: Approximately 4.5 mL filtrate will be collected. Volume of filtrate collected is not critical because g sample/mL acetonitrile is calculated from information in sec. **29.011(f)** (1). If solution needs to be diluted, pipet aliquot into another container and dilute to volume as needed.

Determination

Inject 10 μL acetonitrile sample solution onto HPLC column by using chromatographic apparatus and parameters as described. Tentatively identify quinomethionate peak on basis of retention time. Measure peak area or peak height and determine amount of residue by comparison with peak area or peak height obtained from known amount of reference material. To ensure valid measurement of residue amount, size of peak from sample residue and reference standard should match within ±25%. Chromatograph quinomethionate standard immediately after sample.

Results and Discussion

HPLC-Fluorescence Studies

The fluorescence characteristics of quinomethionate were studied in acetonitrile–water (1 + 1) and acetonitrile–aqueous 0.04N NaOH (1 + 1), using a Perkin-Elmer Model MPF-44A spectrofluorometer. Quinomethionate fluoresced in the neutral solvent but not in base. The spectra obtained (ratio mode) showed a maximum excitation and emission at 360 and 394 nm, respectively. With the HPLC fluorescence detector, the maximum excitation and emission were 362 and 395 nm, respectively, indicating good experimental agreement between the 2 instruments.

The HPLC studies were conducted with the same apparatus and system configuration as that used for the HPLC post-column fluorometric labeling determination of *N*-methylcarbamate insecticides (2). The difference was that no post-column reagents were added and the hydrolysis chamber heater was turned off. Utilizing the HPLC parameters given under *Method*, which were previously used for other fluorescent pesticides (3), quinomethionate retention and response data were obtained (Figures 1 and 2). Four ng quinomethionate produced a peak of approximately 50% full-scale recorder deflection and the linear response ranged from 0.5 to 12 ng.

Of 39 other pesticides/metabolities found to fluoresce naturally in neutral solution (15), none has the same fluorescence or HPLC retention properties as quinomethionate on the C-8 column used. Coumaphos (excitation 320, emission 446, retention relative to carbofuran (R_c) 1.62) is the only compound studied that has fluorescence and retention properties approaching those of quinomethionate (excitation 362, emission 395, R_c 1.49), and these compounds are adequately

Table 1. Comparison of 2 solvent systems for eluting quinomethionate from Florisil[a]

Eluate	Ethyl ether–petroleum ether		Acetonitrile–methylene chloride–hexane	
	Solvent ratio	Quinomethionate recovered, %	Solvent ratio	Quinomethionate recovered, %
1	6/94	0	0/20/80	0
2	15/85	51	0.35/50/49.65	102[b]
3	50/50	8	1.5/50/48.5	0
4	100/0	0		

[a] Single determinations.
[b] In subsequent work with a second lot of Florisil, quinomethionate eluted in second and third eluates.

separated by the gradient mobile phase described under *Method*. Thus, HPLC in conjunction with the fluorescence detector appears to provide the desired sensitivity and selectivity.

Multiresidue Method Studies

The multiresidue method used for the determination of *N*-methylcarbamate insecticides (2) was initially studied for applicability to the determination of quinomethionate. In this method methanol is used to extract the pesticides. Unfortunately, methanol totally degraded quinomethionate (1 μg/mL) within 24 h, as demonstrated by the lack of HPLC response. When quinomethionate was dissolved in methanol, the solution turned from colorless to yellow within minutes, indicating degradation of the pesticide. Quinomethionate dissolved in acetonitrile or acetone did not exhibit reduced HPLC response after 24 h and solutions remained colorless. The AOAC multiresidue method for organohalogen and organophosphorus insecticides (1) uses acetonitrile rather than methanol to extract the pesticides and therefore was investigated for applicability to the determination of quinomethionate.

Quinomethionate was not quantitatively recovered from the Florisil cleanup procedure with the ethyl ether–petroleum ether eluting system (sec. **29.015** (1)). However, quinomethionate was quantitatively recovered from the adsorbent with the methylene chloride–acetonitrile–hexane eluting system (sec. **29.033** (14)). A summary of the data with the 2 solvent systems is presented in Table 1.

Recovery Studies

Quinomethionate recovery studies were conducted using the AOAC method with the Florisil solvent eluting system (sec. **29.033** (14)). Oranges were fortified with quinomethionate at the 0.05 and 0.5 ppm levels and apples at the 0.05 ppm level. Ten mL of an acetonitrile fortification solution was added to 100 g crop in a blender jar, followed by 190 mL acetonitrile just before blending the sample. A crop control and duplicate or triplicate fortified crop samples were analyzed. Each set of samples was subjected to the extraction and coextractive removal steps within an 8 h working day. Subsequently, HPLC was used to determine the recovery of quinomethionate through the method. No quinomethionate was found in the control samples (see Figure 2). The recovery of quinomethionate from oranges fortified at the 0.05 and 0.5 ppm levels was 98 and 104% and 93, 102, and 110%, respectively. The recovery from apples fortified at the 0.05 level was 92 and 108%. The overall average recovery was 100% and ranged from 92 to 110%.

Quantitative recovery of quinomethionate through the procedure outlined is only one aspect to be considered in evaluating its applicability to quinomethionate; another important aspect is selectivity. Other fluorescing pesticides/metabolites do not interfere (15) and no interference from apples and oranges was observed in this study. Chromatograms of the apple and orange controls and quinomethionate standard are shown in Figure 2. No extraneous chromatographic peaks were observed in the samples examined.

Conclusions

The basic AOAC multiresidue method (1) for nonfatty foods with the Florisil eluting system (sec. **29.033**) coupled with the HPLC-fluorometric determinative system is applicable to the detection of quinomethionate in apples and oranges. The extension of the AOAC multiresidue method to incorporate HPLC as a determinative technique should stimulate research

activity to expand use of the method for the relatively nonpolar pesticides not amenable to determination by GLC.

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Analysis of Pesticide Residues by Chemical Derivatization. VI. Analysis of Ten Acid Herbicides in Sediment[1]

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A sensitive gas-liquid chromatographic (GLC) method was developed for multiresidue analysis of 10 acid herbicides in sediment. An acidified sediment sample was extracted 3 times with acetone-hexane. The combined organic extract was partitioned with acidified water to remove aqueous co-extractives. To produce low detection limits for all herbicides including MCPA and MCPB for routine monitoring purposes, pentafluorobenzyl esters were formed before GLC analysis. Recoveries of all herbicides from fortified sediment samples were better than 80% at the levels studied. Practical detection limits of this method were 10-25 μg/kg (ppb).

It is well known that sediment is an important aspect of water quality because it adsorbs many pollutants. Although the mechanism for adsorption and desorption of pollutants in the sediment is not well understood, there is no doubt that sediment exerts certain influences on the water quality. Recently, it was pointed out (1) that "as a means of enhancing future water quality studies it is, therefore, useful to emphasize the value of sediment-related information in studies of the aquatic environment." Therefore, analytical methods are needed to determine known or potential pollutants in sediment. Specifically, a sensitive, multiresidue method is needed for the simultaneous analysis of the common acid herbicides in sediment.

A recent article by Sirons, Chau, and Smith (2) indicated that, although some methods are available for determining acid herbicides in soil and sediment, most of these methods are limited to 1-3 herbicides and only a few are developed for 4-5 acid herbicides. At the same time, most of the methods were developed for the detection of herbicides at ppm levels. Previously, Chau and Terry (3, 4) investigated the formation of methyl, 2-chloroethyl, and pentafluorobenzyl (PFB) esters of 10 commonly used acid herbicides (Table 1). Later, Agemian and Chau (5) reported a method for determining low levels of MCPA and MCPB in water by derivatization with pentafluorobenzyl bromide (PFBBr). Subsequently, the method was extended (6) to the multiresidue analysis of 8 acid herbicides in natural water by the formation of 2-chloroethyl and PFB esters. The work on MPCA and MCPB inspired Sattar et al. in 1976 (7) and 1979 (8) to develop a procedure for the determination of MCPA in soil and Cotterill in 1979 (9) to extend the application of PFBBr reagent for the determination of MCPA, MCPB, and mecoprop in soil.

As yet, no sensitive, multiresidue method exists for the simultaneous analysis of 10 acid herbicides in sediment. In this paper, we report a procedure for such analysis with practical detection limits as low as 10-25 μg/kg (ppb) for all herbicides.

Experimental

Apparatus

(a) *Gas chromatograph.*—Model 5710A equipped with ^{63}Ni electron capture detector and Model 7672A automatic liquid sampler (Hewlett-Packard, Avondale, PA, 19311). Operating conditions: temperatures (°C)—injection port 250, column 200, detector 300; argon-methane carrier gas (95 + 5) flow rate 30 mL/min.

(b) *GLC columns.*—1.8 m × 2 mm id coiled glass packed with Ultrabond 20M, 80-100 mesh (Ultra Scientific Inc., Hope, RI 02831); 3% OV-1 on 100-120 mesh Gas-Chrom Q; 3% OV-17 on 80-100 mesh Chromosorb W (HP); and 1.5% OV-17 + 1.95% OV-210 on 100-120 mesh Gas-Chrom Q (Chromatographic Specialities Ltd, Brockville, Ontario, Canada K6V 5W1).

(c) *Sonicator cell disruptor.*—Model 375 W (Heat Systems—Ultrasonics Inc., 38 E Mall, Plainview, NY 11803).

(d) *Filtration apparatus.*—Modified coarse (70-100 μm) sintered glass funnel, 100 × 40 mm id, with standard taper joint and suction sidearm. (Original equipment supplied by Ace Glass Inc., Vineland, NJ 08360.)

(e) *Evaporating apparatus.*—Buchi rotary evaporator with thermostated bath (Fisher Scientific Co. Ltd, Don Mills, Ontario, Canada M3A 1A9).

(f) *Chromatographic columns.*—400 × 20 mm id

[1] For Part V, see *J. Assoc. Off. Anal. Chem.* (1977) **60**, 1070.
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Table 1. Common and chemical names of 10 acid herbicides and practical detection limits in sediment samples

Common name	Chemical name	Detection limit,[a] ng/g
Dicamba	2-methoxy-3,6-dichlorobenzoic acid	10
2,3,6-TBA	2,3,6-trichlorobenzoic acid	10
2,4-DP	2-(2,4-dichlorophenoxy)-propionic acid	10
MCPA	2-methyl-4-chlorophenoxyacetic acid	10
Silvex	2-(2,4,5-trichlorophenoxy)-propionic acid	10
2,4-D	2,4-dichlorophenoxyacetic acid	10
MCPB	4-(2-methyl-4-chlorophenoxy)-butyric acid	25
2,4,5-T	2,4,5-trichlorophenoxyacetic acid	25
2,4-DB	2,4-dichlorophenoxybutyric acid	25
Picloram	4-amino-3,5,6-trichloropicolinic acid	25

[a] Based on 5 g sample and final volume of extract adjusted to 8 mL.

with coarse fritted disc and Teflon stopcock (Fisher Scientific Co. Ltd).

(g) *Vortex Genie.*—Test tube mixer (Fisher Scientific Co. Ltd).

(i) *Tube heater.*—Model K-720002 (Kontes, Vineland, NJ 08360).

Reagents

Use pesticide grade solvents and reagent grade chemicals. Check each batch for interferences before use.

(a) *Sodium sulfate.*—Anhydrous reagent grade (BDH Chemicals, Toronto, Ontario, Canada M8Z 1K5). Heat 18 h at 650°C and store in glass containers.

(b) *30% K_2CO_3 solution.*—Dissolve 30 g anhydrous K_2CO_3, ACS grade, in 100 mL water.

(c) *Pentafluorobenzyl bromide (PFBBr) reagent (α-bromo-2,3,4,5,6-pentafluorotoluene).*—Transfer 5 mL reagent (Aldrich Chemical Co., Milwaukee, WI 53233) to low actinic 100 mL volumetric flask and dilute to volume with acetone. (Caution: Reagent is strong lachrymator.)

(d) *Silica gel.*—Grade 950 for gas chromatography, 60–200 mesh (Fisher Scientific Co.). Activate by heating overnight (14 h) at 130°C. Deactivate by adding 5 mL pure water to 95 g activated silica gel. Mix well by tumbling overnight in a tightly capped glass container before use. Prepare fresh weekly.

(e) *Herbicide standards.*—Analytical grade (98+% pure) were obtained from manufacturers or U.S. Environmental Protection Agency (HERL, Research Triangle Park, NC 27711) and were used as received.

(f) *Pure water.*—Prepare according to ref. 10 or pass distilled water through Millipore Super-Q unit (Millipore Corp., Bedford, MA 01730).

(g) *Acidified organic-free water.*—Add 2 mL H_2SO_4 solution (1 + 1) to 1 L pure water. Extract solution with 50 mL dichloromethane for 30 min by stirring. Discard organic layer.

Extraction

Weigh 50 g sample of a homogeneous wet sediment into 250 mL beaker. Concurrently, weigh another sample into tared beaker for moisture content determination and dry at 105°C to constant weight. If necessary, wet sample in beaker with organic-free water to an estimated 30% moisture content and mix thoroughly with a spatula.

Carefully acidify sediment to pH ≤ 1 with H_2SO_4 solution (1 + 1) (about 10 mL). Let mixture stand 20 min and stir occasionally. Then add 100 mL acetone–hexane (1 + 1). Immerse ultrasonic cell disruptor horn 2 cm into sample. Activate sonicator in pulsed mode at 50% duty cycle with maximum output. After 3 min, stop sonication and let sediment settle.

Prepare 5 cm Celite column in sintered glass filter funnel. Wash column with 100 mL acetone–hexane (1 + 1). Activate vacuum to remove solvent from Celite bed. Discard solvent. Decant sediment extract into Celite column, apply vacuum, and collect filtrate in clean, round-bottom flask.

Repeat extraction by sonication with two 100 mL portions of same solvent, filter as described, and collect all filtrates in same round-bottom flask. After last filtration, transfer whole sediment sample from beaker to Celite column. Apply vacuum until column is nearly dry. Rinse beaker with 20 mL extraction solvent and filter rinse through Celite column with vacuum as above.

Transfer combined filtered extract, after rinsing with two 10 mL portions of acetone–hexane (1 + 1), into 500 mL separatory funnel containing acidified organic-free water (100 mL). Shake 1 min and vent frequently. Let layers separate. Transfer aqueous (lower) layer back to original round-bottom flask.

Carefully drain organic layer from separatory funnel into clean, dry round-bottom flask, taking care not to let any water enter flask. Transfer aqueous layer back to separatory funnels. Rinse

flask with 50 mL dichloromethane and pour rinsing into above funnel. Shake contents 1 min. Let layers separate. If emulsion persists, leave it with aqueous layer. Drain organic (lower) layer into previous round-bottom flask containing first extract. Again avoid getting water into the organic phase.

Repeat extraction of aqueous phase with two 50 mL portions of dichloromethane as described above. After last extraction, discard aqueous layer.

Evaporate combined organic extracts in round-bottom flask to about 20 mL, using rotary evaporator, reduced pressure, and 40°C water bath. Add 50 mL benzene and repeat rotary evaporation until just to dryness (see discussion). Redissolve residue in four 2 mL portions of acetone. Transfer solution with disposable pipet to 15 mL graduated centrifuge tube. Wash pipet into tube with 1 mL acetone. Dilute to 10.0 mL with acetone. Mix well.

Esterification

Transfer 1.0 mL of the above acetone solution to clean centrifuge tube. If more acetone extract is desirable, record exact volume used. In all cases, adjust acetone volume to 4 mL by evaporation (nitrogen stream at 30–40°C) or by dilution.

Add 30 μL 30% K_2CO_3 solution and 200 μL PFBBr reagent. Close tube with glass stopper. Mix contents on Vortex Genie. Heat tube in tube heater at 60°C for 3 h, making sure stopper is tightly in place. (Wrap thin Teflon tape around stopper once or twice and insert stopper with twist into tube action.)

After reaction, evaporate solution (no need for cooling) to 0.5 mL with gentle stream of nitrogen. (Add 2 mL hexane and repeat evaporation just to dryness at room temperature (see discussion). Redissolve residue in 2 mL benzene–hexane (1 + 9) for column cleanup.

Column Cleanup

Prepare mini-column by plugging 20 cm long Pasteur pipet with piece of silanized glass wool and filling it with 5 cm of 5% deactivated silica gel topped with 0.5 cm of anhydrous Na_2SO_4.

Prewet column with hexane (5 mL), and let solvent drain just to top of adsorbent. Quantitatively transfer to column the above concentrated sample extract with several separate rinsings (total 2–3 mL) of benzene-hexane (1 + 9). Elute column with the same solvent to collect 8 mL. Discard this fraction which contains excess reagent.

Elute same column with benzene-hexane (75 + 25) to collect 3 mL which contains PFB derivatives of dicamba, 2,3,6-TBA, 2,4-DP, MCPA, silvex, 2,4-D, MCPB, 2,4,5-T, and 2,4-DB (fraction 1).

Elute same column with ether-benzene (1 + 9) to collect 8 mL which contains PFB derivative of picloram (fraction 2).

Record volume of both fractions 1 and 2. Analyze these fractions by electron capture gas-liquid chromatography (EC-GLC). If levels of derivatives are too high, appropriate dilution before EC-GLC may be necessary. Concentration by evaporation is not recommended before GLC examination because of interference from by-products of PFB reaction.

Results and Discussion

The choice of acetone–hexane (1 + 1) as extraction solvent is based on its wide applicability to the extraction of several classes of organic compounds from sediments, for example, PCBs (10), organochlorines, chlorobenzenes, as well as neutral herbicides such as triazine, urea, and carbamates (11, and unpublished results). The use of a more polar solvent system such as acetone does not give higher recoveries of the acid herbicides. Moreover, if acetone is used instead of the chosen solvent mixture for the extraction of the acidified sediments, an emulsion may be formed in the partitioning step if the sediment is high in humic and organic contents.

As reported previously (6), to achieve an effective multiresidue extraction of acid herbicides from an aqueous matrix, the sample has to be adjusted to pH $\leq$ 1. The sediment samples, containing at least 30% water, were therefore acidified for maximum extraction efficiency.

An acid-base partitioning step for the cleanup of sediment extract is not described in the procedure because partitioning did not provide any significant reduction of sample co-extractives on all types of sediment tested. Instead of the above, the sediment extract is partitioned with acidified water to remove water and inorganic acid while leaving the herbicides in the organic phase. However, if the analyses of neutral herbicides and other neutral compounds are included in this procedure, the acid-base partitioning step is obviously necessary.

The use of anhydrous sodium sulfate, even acidified (12), is not incorporated into the procedure for drying the sample extract. Use of this drying agent led to erratic recoveries of acid herbicides. The last trace of water remaining in the sample extract after partitioning can be ef-

fectively removed by azeotropic distillation in the presence of benzene.

It is well known that acid herbicides have to be derivatized before they can be analyzed by GLC methods. The most popular approach is the formation of methyl esters by a methylating agent such as BF_3/methanol or CH_2N_2. However, BF_3/methanol does not methylate dicamba and 2,3,6-TBA. Although CH_2N_2 reacts with all 10 herbicides to form methyl esters, this reagent is known to be unstable and explosive. Also, the very low ECD response of MCPA and MCPB methyl esters make these derivatives not generally applicable for water analysis. Thus, the formation of methyl esters is not desirable for the multiresidue purpose. One can form the more sensitive 2-chloroethyl esters but dicamba and 2,3,6-TBA do not form these readily (3) by the BCl_3 method. On the other hand, the electron capture detector is sensitive to all the pentafluorobenzyl (PFB) derivatives so that a low detection limit can be established for all 10 herbicides including MCPA and MCPB. Moreover, the PFB esters of all herbicides can be easily formed and the reagents are readily available and stable.

Because of the detector sensitivity to the PFB esters formed, only 10% of the total sediment extract is required in the esterification step to achieve the detection limits set out in Table 1. The remaining sediment extract can be used to form other derivatives such as the methyl esters if further confirmation of identities is required.

As reported earlier (4), the reaction volume and medium of the PFBBr esterification is important for maximum yield of the herbicide PFB derivatives. It was observed in this study that the rate of formation of many PFB esters is much slower if the polarity of the reaction mixture is lower (i.e., when acetone–hexane (1 + 1) is used instead of pure acetone, and solid K_2CO_3 is used instead of 30% K_2CO_3 solution). The same is observed when large amounts of naturally occurring sediment co-extractives are present in the extract. Presumably, the high humic content in the sample extract competes with acid herbicides for the reagents so that the desired reaction is slowed. Also, it is not desirable to have a large amount of water in the reaction mixture because, under the reaction conditions, water is known to form dipentafluorobenzyl ether and other side products which may interfere with the GLC analysis (4, 6, 13). For best results, esterification of sediment extracts should be carried out in a total of 4 mL acetone with 200 μL 5% PFBBr reagent and 30 μL 30% K_2CO_3 solution at 60°C for 3 h rather than at room temperature for 5 h for standard solutions (4) or at room temperature overnight for water extracts (5, 6).

Table 2. Retention times (min) of acid herbicide–PFB esters on different columns

Herbicide	Ultrabond 20M, 190°C	3% OV-1, 180°C	3% OV-17, 200°C	1.5% OV-17 + 1.95% OV-210, 200°C
Dicamba	5.01	6.79[a]	4.80	6.14
2,3,6-TBA	5.68	7.56[b]	5.52	6.93[a]
2,4-DP	6.12	7.56[b]	5.16[a]	6.93[a]
MCPA	7.16	6.79[a]	5.16[a]	6.93[a]
Silvex	9.56	12.37	8.54	10.76
2,4-D	11.49	9.00	7.18	9.31
MCPB	16.13	17.52	12.30[b]	15.60
2,4,5-T	19.38	15.14	12.30[b]	15.09
2,4-DB	22.15	22.04	16.93	20.68
Picloram	127.46	30.00	37.20	42.42

[a,b]Unresolved pairs. Flow rates ca 30 mL/min in each case.

Basically, the same post-esterification column cleanup procedure previously described for 9 herbicides in water samples (6) was used here for the sediments. However, to include 2,3,6-TBA in the procedure, 8 mL of 10% rather than 25% benzene in hexane should be used for the removal of excessive reagents and interfering materials.

The resolution of methyl and PFB esters of 10 phenoxyalkanoic acid herbicides on 9 commonly used liquid phases was studied by DeBeer et al. (14). They found that the 10 PFB esters were completely separated on the apolar phases DC-200 and DC-11, and on the polar phase FFAP with a different elution order. The methyl esters, on the other hand, were completely separated with the same elution order on the semipolar phases OV-17, XE-60, and OV-225. However, their study did not include dicamba, 2,3,6-TBA, and picloram. In this study which includes the above three and 7 other phenoxyalkanoic acid herbicides, complete separation of the 10 PFB derivatives can only be obtained with the polar Ultrabond 20M column but not with the apolar OV-1, semipolar OV-17 and OV-225, or mixed phase OV-101/OV-210 and OV-17/OV-210 columns (see Table 2 and ref. 4). Because of the resolution of the Ultrabond 20M column, it is the column of choice for the analysis of acid herbicide PFB esters in sediment samples. This column has a lifetime of at least 3 months of continuous operation. It should be noted that picloram PFB ester has an extremely long retention time on the Ultrabond column, thus it is

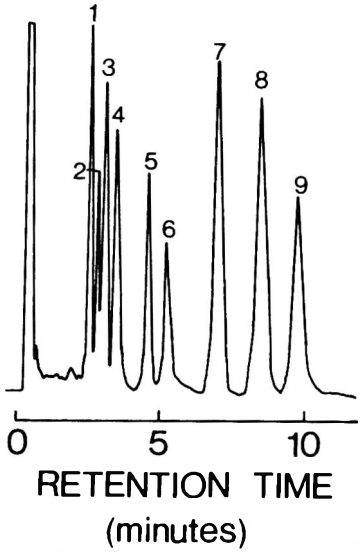

Figure 1. Gas chromatogram of standard mixture of 9 acid herbicide PFB esters on Ultrabond 20M column at attenuation 128; 5.0 μL injected. (1) 25 pg Dicamba, (2) 25 pg 2,3,6-TBA, (3) 25 pg 2,4-DP, (4) 25 pg MCPA, (5) 25 pg Silvex, (6) 25 pg 2,4-D, (7) 63 pg MCPB, (8) 63 pg 2,4,5-T, (9) 63 pg 2,4-DB. For other conditions, see *Experimental*.

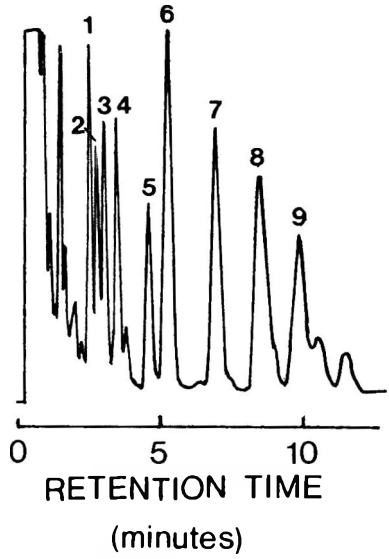

Figure 2. Chromatogram of extract derived from sediment sample fortified at detection limit. For peak identification, see Figure 1. For fortification level of each herbicide, see Table 3.

more convenient to analyze this compound (the only component in fraction 3 after the mini-silica gel column cleanup) by the other columns listed in Table 2. Although the OV-1, OV-17, and OV-17/OV-210 columns have one or 2 unresolved pairs of PFB esters, combinations of these columns can be used for the confirmation of herbicides identities. A chromatogram of a standard mixture of the 9 herbicide PFB esters in fraction 2 is depicted in Figure 1.

In this study, 3 types of river sediments from the Battle River, Old Man River, and Red Deer River in the prairie provinces were used for re-

Table 3. Percent recovery and precision of analysis of 10 acid herbicides in fortified sediments [a]

Herbicide	Level of spiking, μg/kg (ppb)	% Recovery ± SD		
		Sediment A (n = 3)	Sediment B (n = 6)	Sediment C (n = 3)
Dicamba	100	91.3 ± 3.1	87.0 ± 5.0	94.3 ± 5.5
	10	94.3 ± 6.4	85.7 ± 3.1	96.3 ± 12.0
2,3,6-TBA	100	84.3 ± 2.9	91.2 ± 9.2	105.3 ± 6.4
	10	92.7 ± 3.2	106.8 ± 19.3	102.0 ± 18.0
2,4-DP	100	94.7 ± 2.5	85.8 ± 4.5	87.0 ± 4.4
	10	83.3 ± 5.9	87.5 ± 10.9	85.3 ± 7.5
MCPA	100	105.3 ± 2.5	87.3 ± 4.8	90.7 ± 3.2
	10	88.7 ± 2.1	88.5 ± 13.0	79.7 ± 4.5
Silvex	100	103.3 ± 2.1	92.0 ± 3.5	89.0 ± 4.4
	10	91.7 ± 5.4	80.8 ± 7.7	84.3 ± 9.5
2,4-D	100	93.7 ± 4.7	89.8 ± 5.7	101.3 ± 4.0
	10	107.7 ± 6.0	84.3 ± 4.0	90.7 ± 1.5
MCPB	250	100.0 ± 3.6	91.7 ± 5.7	70.7 ± 4.0
	25	89.3 ± 6.0	88.2 ± 13.6	73.7 ± 6.8
2,4,5-T	250	108.7 ± 3.5	88.5 ± 7.7	89.3 ± 4.5
	25	88.7 ± 5.1	88.7 ± 10.7	83.0 ± 6.6
2,4-DB	250	79.7 ± 8.3	95.0 ± 5.2	78.7 ± 3.2
	25	103.0 ± 12.0	90.0 ± 9.9	80.7 ± 7.8
Picloram	250	84.7 ± 2.5	81.7 ± 7.0	86.0 ± 6.6
	25	76.0 ± 5.2	79.7 ± 4.2	81.7 ± 3.5

[a] Sediment A: Battle R. sediment; Sediment B: Old Man R. sediment; Sediment C: Red Deer R. sediment.

covery experiments. These sediments contained considerably more organic matter than did Lake Superior sediment and their texture varies from sandy loam to silty clay. The recoveries of herbicides in the fortified samples at 2 concentration levels are given in Table 3. The detection limits (Table 1) apply to the types of sediments used in our experiments and they may become a little higher or lower depending on the sample blank of other sediments. A typical chromatogram of an extract derived from a sediment sample fortified at 10–25 ppb is shown in Figure 2.

Conclusion

To develop a multiresidue analytical method for 10 acid herbicides in sediments with a low enough detection limit for routine surveillance and monitoring for our regional Water Quality Laboratories, PFBBr esterification is the only suitable choice. As discussed earlier, alkylation including methylation and 2-chloroethylation is not suitable due to the ECD sensitivity and GLC column resolution of the derivatives formed does not meet the requirements for the multiresidue analysis of these 10 acid herbicides in sediments.

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Analysis of Pesticide Residues by Chemical Derivatization. VII. Chromatographic Properties of Pentafluorobenzyl Ether Derivatives of Thirty-two Phenols[1]

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A gas-liquid chromatographic (GLC) procedure is described for the identification of 32 substituted phenols including all 19 chlorophenols and the 11 U.S. Environmental Protection Agency consent decree phenols. This method involves a simple and reproducible derivatization step which forms stable phenol PFB ethers for which the electron capture detector is highly sensitive. GLC retention time data of the derivatives on 6 packed and fused silica capillary columns (FSCC) are reported. The detection limits of all chloro-, alkyl- and mono-nitrophenols studied are between 0.5 and 5 pg injected for an FSCC. The high resolution of these capillary columns makes this method isomer-specific. The derivatization procedure also eliminates the sensitivity, tailing, and resolution problems commonly encountered in other gas chromatographic methods on underivatized phenols.

Phenols are a major class of industrial chemicals. They are also precursors and degradation products of many pesticides. In particular, chlorophenols are widely used in the synthesis of herbicides, and PCP is often used as a wood preservative. Indeed, many chlorophenols are toxic. The recent interest in the analysis of chlorophenols is due partly to the presence of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in the mixtures of chlorophenols. Chlorophenols also have an adverse effect on the odor and taste of drinking water at low ppb levels. Some of the above aspects on chlorophenols have been discussed in ref. 1.

Because of the large number of phenols and their different toxicities, it is essential to have an analytical method which is isomer-specific and yet sensitive to all phenols. Existing methodologies were developed either for a limited number of phenols or for relatively high levels. For environmental analysis, low level detection (parts per billion or lower) for toxic organic compounds are often required. Before an analytical method for a large number of phenols is to be developed, it is necessary to establish the derivatization procedure and chromatographic conditions for these derivatives. In this paper, we present a suitable gas and column chromatographic procedure for the identification of 32 phenols (Table 1) including all 19 chlorophenols and 11 U.S. Environmental Protection Agency priority phenols (2) as their pentafluorobenzyl ether derivatives.

Experimental

Gas Chromatographs

(*1*) Hewlett-Packard Model 5713 A equipped with ^{63}Ni electron capture detector. Model 7671A Autosampler and Model 3390A reporting integrator. Operating temperatures: injection port 250°C, detector 300°C, column, see below. Carrier gas methane–argon (5 + 95).

(*2*) Hewlett-Packard Model 5880A equipped with ^{63}Ni electron capture detector, Model 7671A Autosampler, Level Four terminal, and split-splitless capillary column injection port. Operating temperatures: injection port 250°C, detector 300°C, column, see below. Carrier gas, helium. Both instruments and accessories are available from Hewlett-Packard, Avondale, PA 19311.

Columns

(*1*) 1.8 m × 2 mm id glass packed with 3% OV-1 on 100–120 mesh Gas-Chrom (Chromatographic Specialities Ltd, Brockville, Ontario, Canada, K6V 5W1); temperature 165°C; flow rate 28 mL/min.

(2) 1.8 m × 2 mm id glass packed with 3% OV-17 on 120–140 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, PA 16801); temperature 170°C, flow rate 25 mL/min.

(3) 1.8 m × 2 mm id glass packed with Ultrabond 20M, 80–100 mesh (Ultra Scientific Inc., Hope, RI 02831); temperature 155°C; flow rate 26 mL/min.

(4) 12 m × 0.2 mm id fused silica capillary column (FSCC) coated with methyl silicone fluid and surface-deactivated by Carbowax (Part No.

[1] For Part VI, see *J. Assoc. Off. Anal. Chem.* (1983) **66**, 1023.
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19091-60010, Hewlett-Packard). Temperatures: initial 70°C, hold for 0.5 min; programming rate 1, 25°/min (70 to 160°C), rate 2, 2°/min (160 to 200°C); final time 10 min. Splitless valve on for 30 s; linear velocity 25 cm/s.

(5) 12 m × 0.2 mm id FSCC coated with and deactivated by Carbowax 20M (Part No. 19091-60125, Hewlett-Packard). Temperatures and conditions are the same as column 4; linear velocity 27 cm/s.

(6) 30 m × 0.25 mm id FSCC coated with SE-54, film thickness 0.25 μm, and surface-deactivated by siloxane (J&W Scientific Inc., Rancho Cordova, CA 95670). Temperatures: initial 70°C, hold for 0.5 min; programming rate 1, 25°/min (70 to 180°C), rate 2, 2°/min (180 to 220°); final time 10 min. Splitless valve on for 30 s; linear velocity 23 cm/s.

Reagents

Use all pesticide grade solvents.

(1) *Phenols.* Obtained from Aldrich Chemical Co. (PO Box 355, Milwaukee, WI 53201) or Supelco, Inc. (Phenol Kit 27, Bellefonte, PA 16823). 2,3,4,6-Tetrachlorophenol obtained from Eastman Organic Chemicals (Rochester, NY 14650). Prepare all stock solutions in benzene–isooctane (10 + 90) at 1 mg/mL. Keep in dark at 4°C.

(2) *Pentafluorobenzyl bromide (PFBBr) reagent.* Dissolve 5 g PFBBr (Aldrich Chemical Co.) in acetone and dilute to 100 mL with the same solvent.

(3) *30% K_2CO_3 solution.* Dissolve 30 g K_2CO_3 (anhydrous) in water and dilute to 100 mL.

Microsynthesis of Phenol PFB Ethers

To 4 mL acetone solution of phenol or mixture of phenols (total phenol content less than 100 μg) in test tube, add 100 μL 5% PFBBr reagent and 25 μL of 30% K_2CO_3 solution. Stopper tube and mix well. Heat mixture at 60°C in a test tube heater for 30 min. After heating, carefully evaporate solution to 0.2 mL, using gentle stream of nitrogen. Add 1 mL hexane, mix well, and evaporate to 0.2 mL again. Note: For higher yields of the dinitrophenol PFB ethers, heat mixture at 60°C for 5 h instead of 30 min (see discussion below).

Cleanup

Prepare a mini cleanup column by plugging a long Pasteur pipet (23 × 0.5 cm id) with piece of silanized glass wool. Fill column with 5 cm 5% deactivated silica gel. Tap column gently and add 5 mm anhydrous Na_2SO_4 at top.

Prewet column with 5 mL hexane and discard rinsings. With Pasteur pipet, quantitatively transfer concentrated sample extract in hexane plus hexane rinsings from test tube to silica gel column. Elute fractions as follows:

Fraction 1: Elute column with benzene–hexane (25 + 75) and collect 8 mL. This fraction contains the PFB ethers of phenols 1–26 (Table 1), namely, all 19 chlorophenols, chloroalkylphenols, alkylphenols, thiophenol, and phenol.

Fraction 2: Elute column with benzene–hexane (75 + 25) and collect 8 mL. This fraction contains the PFB ethers of phenols 27–30, namely, the mono-nitro phenols and cyanophenol.

Fraction 3: Elute column with methanol–benzene (5 + 95) and collect 8 mL. This fraction contains the PFB ethers of the dinitro phenols (Nos. 31 and 32).

Results and Discussion

Choice of Phenols

The choice of phenols for our investigation was based on the following considerations:

Many chlorophenols are toxic substances present either in biocidal formulations, as industrial pollutants, or as degradation products of pesticides such as *N*-methyl carbamates, organophosphorus pesticides, and phenoxyalkanoic herbicides. Although not all 19 chlorophenols are equally toxic or of equal environmental importance, it is necessary to know the analytical characteristics (e.g., GLC retention times) of all these phenols to establish any possible cross-interference during the analysis. This knowledge will provide information on the limitation or ruggedness of any analytical method based on this derivatization technique.

Also included in this work are all other EPA priority phenols (2) which are not already included above. Seven of the phenols (Nos. 7, 13, 25, 26, 27, 29, and 30) are the hydrolyzed products of 8 organophosphorus pesticides for which the identities were confirmed by chemical derivatization with PFBBr (3, 4). The remaining miscellaneous phenols were requested by our Analytical Services Laboratories in Moncton and Burlington. Inclusion of all these phenols widens the scope of the application of this screening method.

Analysis

Phenols have been analyzed by a variety of methods. Un-derivatized phenols can be analyzed by HPLC by using a reverse phase column

Table 1. Retention times (min) and relative retention times (PCP PFB ether = 10.00) of 32 phenol PFB ethers on 6 columns [a]

No.	Parent phenol	3% OV-1	3% OV-17	Ultrabond 20M	OV-101 FSCC	SE-54 FSCC	Carbowax 20M FSCC
1 [b]	Phenol	1.30 (0.54)	1.17 (0.43)	1.14 (0.57)	5.07 (2.45)	8.96 (3.04)	4.74 (2.66)
2 [b]	2-Chloro-	2.30 (0.95)	2.25 (0.83)	2.26 (1.14)	6.54 (3.16)	11.28 (3.83)	6.45 (3.63)
3	3-Chloro-	2.59 (1.07)	2.42 (0.90)	2.55 (1.28)	6.86 (3.32)	11.66 (3.96)	6.73 (3.78)
4	4-Chloro-	2.74 (1.13)	2.60 (0.96)	2.84 (1.43)	7.05 (3.41)	11.96 (4.05)	7.10 (3.99)
5	2,6-Dichloro-	3.56 (1.47)	3.42 (1.27)	2.95 (1.48)	8.03 (3.89)	13.49 (4.58)	7.27 (4.09)
6	2,5-Dichloro-	4.08 (1.68)	4.28 (1.59)	4.59 (2.31)	8.68 (4.20)	14.30 (4.85)	8.86 (4.98)
7 [b]	2,4-Dichloro-	4.34 (1.79)	4.36 (1.62)	4.84 (2.44)	8.96 (4.34)	14.68 (4.98)	9.12 (5.13)
8	3,5-Dichloro-	4.49 (1.85)	4.12 (1.53)	4.64 (2.33)	9.09 (4.40)	14.68 (4.98)	8.80 (4.95)
9	2,3-Dichloro-	4.96 (2.05)	5.49 (2.04)	6.26 (3.15)	9.62 (4.65)	15.64 (5.31)	10.42 (5.86)
10	3,4-Dichloro-	5.42 (2.23)	5.61 (2.08)	6.70 (3.37)	10.05 (4.86)	16.06 (5.45)	10.76 (6.05)
11 [b]	2,4,6-Trichloro-	5.84 (2.41)	5.44 (2.02)	4.30 (2.16)	10.37 (5.02)	16.57 (5.62)	8.51 (4.78)
12	2,3,6-Trichloro-	6.74 (2.78)	6.97 (2.58)	6.17 (3.10)	11.20 (5.42)	17.76 (6.03)	10.25 (5.76)
13	2,4,5-Trichloro-	7.89 (3.25)	8.50 (3.15)	9.46 (4.76)	12.17 (5.89)	18.81 (6.38)	12.46 (7.00)
14	2,3,5-Trichloro-	8.05 (3.32)	8.42 (3.12)	9.47 (4.76)	12.20 (5.90)	18.86 (6.40)	12.48 (7.02)
15	2,3,4-Trichloro-	10.03 (4.13)	11.79 (4.37)	14.74 (7.41)	13.79 (6.67)	20.94 (7.11)	15.61 (8.78)
16	3,4,5-Trichloro-	10.31 (4.25)	10.68 (3.96)	13.42 (6.75)	13.90 (6.73)	20.82 (7.07)	14.84 (8.34)
17	2,3,5,6-Tetrachloro-	11.69 (4.82)	11.97 (4.44)	9.40 (4.73)	14.77 (7.15)	22.12 (7.51)	12.46 (7.00)
18	2,3,4,6-Tetrachloro-	12.29 (5.07)	12.54 (4.65)	9.69 (4.87)	15.03 (7.27)	22.45 (7.62)	12.77 (7.18)
19	2,3,4,5-Tetrachloro-	17.24 (7.11)	20.59 (7.63)	24.23 (12.19)	17.80 (8.61)	25.59 (8.68)	19.03 (10.70)
20 [b]	PCP	24.26 (10.00)	26.97 (10.00)	19.88 (10.00)	20.67 (10.00)	29.47 (10.00)	17.79 (10.00)
21 [b]	2,4-Dimethyl-	2.30 (0.95)	2.00 (0.74)	1.74 (0.88)	6.60 (3.19)	11.20 (3.80)	5.39 (3.03)
22	2,3-Dimethyl-	2.64 (1.09)	2.30 (0.85)	1.97 (0.99)	6.95 (3.36)	11.74 (3.98)	5.74 (3.23)
23	2-Chloro-5-methyl-	3.22 (1.33)	3.04 (1.13)	2.98 (1.50)	7.66 (3.71)	12.81 (4.35)	7.11 (4.00)
24 [b]	4-Chloro-3-methyl-	3.86 (1.59)	3.59 (1.33)	3.66 (1.84)	8.38 (4.05)	13.76 (4.67)	7.84 (4.41)
25	2-Chloro-4-tert-butyl-	7.30 (3.01)	6.84 (2.54)	6.05 (3.04)	11.63 (5.63)	17.95 (6.09)	9.07 (5.10)
26	Thiophenol	1.89 (0.78)	1.82 (0.67)	1.54 (0.78)	5.91 (2.86)	10.44 (3.54)	5.36 (3.01)
27	4-Cyano-	5.22 (2.15)	7.57 (2.81)	15.24 (7.67)	9.85 (4.77)	16.10 (5.46)	16.38 (9.21)
28 [b]	2-Nitro-	5.08 (2.09)	7.54 (2.80)	14.10 (7.09)	9.92 (4.80)	16.16 (5.48)	15.82 (8.89)
29 [b]	4-Nitro-	8.29 (3.42)	12.12 (4.49)	24.83 (12.49)	12.62 (6.11)	19.56 (6.64)	20.31 (11.42)
30	3-Methyl-4-nitro-	10.14 (4.18)	14.35 (5.32)	26.44 (13.30)	13.95 (6.75)	20.92 (7.10)	20.15 (11.33)
31 [b]	2,4-Dinitro-	22.45 (9.25)	51.80 (19.21)	— [c]	21.05 (10.18)	29.32 (9.95)	— [d]
32 [b]	4,6-Dinitro-2-methyl-	21.29 (8.78)	37.50 (13.90)	— [c]	19.83 (9.59)	28.38 (9.63)	— [d]

[a] For GLC conditions, see experimental section.
[b] U.S. EPA priority pollutants.
[c] Did not chromatograph at 155°C in 60 min.
[d] Did not chromatograph under the conditions used.

Table 2. Reproducibility of PFBBr derivatization reaction [a] and relative response factors [b] of the derivatives

No.	Parent phenol	RSD, %	Rel. response factor
1	Phenol	7.9	2.28
2	2-Chloro-	6.2	3.97
3	3-Chloro-	6.8	3.18
4	4-Chloro-	7.5	2.79
5	2,6-Dichloro-	4.2	4.23
6	2,5-Dichloro-	5.2	4.68
7	2,4-Dichloro-	6.1	5.26
8	3,5-Dichloro-	5.0	5.13
9	2,3-Dichloro-	3.9	4.40
10	3,4-Dichloro-	4.5	4.06
11	2,4,6-Trichloro-	3.0	5.38
12	2,3,6-Trichloro-	5.6	5.56
13	2,4,5-Trichloro-	4.4	4.62
14	2,3,5-Trichloro-	5.4	3.55
15	2,3,4-Trichloro-	5.2	4.02
16	3,4,5-Trichloro-	3.7	6.09
17	2,3,5,6-Tetrachloro-	2.4	5.50
18	2,3,4,6-Tetrachloro-	4.5	5.70
19	2,3,4,5-Tetrachloro-	3.0	9.01
20	PCP	3.7	10.00
21	2,4-Dimethyl-	5.6	1.78
22	2,3-Dimethyl-	6.3	5.60
23	2-Chloro-5-methyl-	7.7	4.29
24	4-Chloro-3-methyl-	8.5	3.96
25	2-Chloro-4-tert-butyl-	6.5	3.77
26	Thiophenol	9.7	2.54
27	4-Cyano-	—	2.20
28	2-Nitro-	4.5	3.41
29	4-Nitro-	4.5	1.48
30	3-Methyl-4-nitro-	3.8	1.52

[a] Precision of 6 replicate derivatization reactions for phenols at 1–2 μg level.

[b] ECD response factors based on peak areas relative to PCP PFB ether (= 10.00) with OV-101 FSCC. All response factors are not corrected for yields of derivatives.

and a UV detector (5). Free phenols have also been analyzed by gas-liquid chromatography (GLC) with specially deactivated packing materials and either an FID (2) or ECD (6). Although useful in some cases, the high pressure liquid chromatographic (HPLC) and GLC/FID analytical methods for free phenols usually lack the sensitivity required in environmental monitoring and surveillance. The GLC/ECD methods do provide good sensitivities for the dichloro- and higher chlorinated phenols; however, for monochloro- and alkylphenols, the sensitivity is impractically low. More recently, free phenols were also analyzed on fused silica capillary columns (FSCC) with either ECD or FID (7). While these columns provide much improved resolution of the phenols, the problems on sensitivity stated above remained unchanged.

On the other hand, phenols are also analyzed by forming derivatives with reagents such as PFBBr (8–11), HFBI (12), 1-fluoro-2,4-dinitrobenzene (13), acetic anhydride (14). A detailed discussion on various derivatization methods of phenols is given in ref. 8. Although derivatization of phenols requires a longer analytical procedure, the extra time spent is more than offset by the following advantages: (a) Most derivatives improve detector response and thus lower the detection limit. (b) Derivatives require no special column for GLC analysis. In the case of packed column GLC, the chromatography of free phenols requires specially deactivated or bonded column packings to improve peak shape and reduce adsorption (2, 6). With capillary columns, free phenols do not chromatograph on a Carbowax deactivated column which has a basic surface, although the same phenols are chromatographed easily on columns with acidic surfaces (7). For derivatives of phenols, and PFB ethers in particular, there is no such limitation on either packed or capillary column chromatography. (c) Derivatives of phenols can be further cleaned up by column chromatography. This extra cleanup which is extremely useful for analysis at low levels, is generally not amenable to acidic compounds such as phenols. (d) In

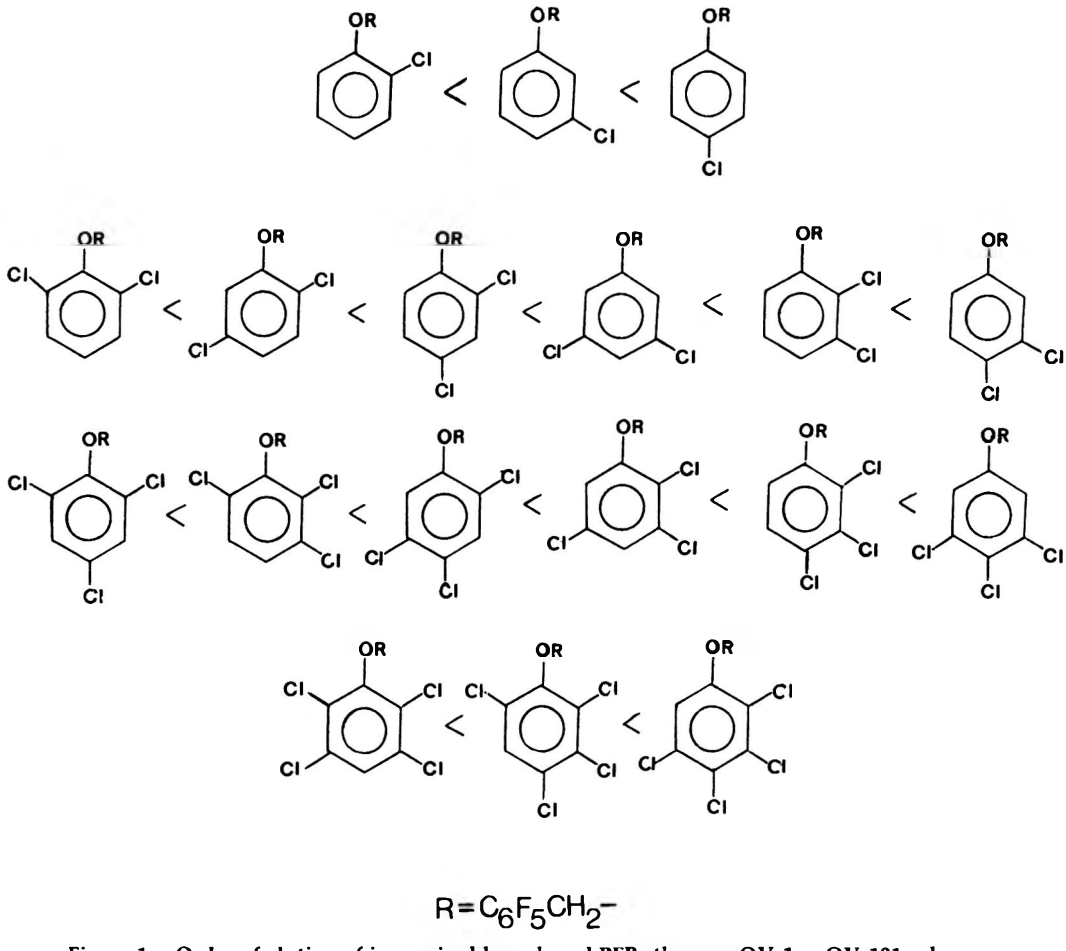

Figure 1. Order of elution of isomeric chlorophenol PFB ethers on OV-1 or OV-101 columns.

many cases, derivatives are better resolved than their parent phenols by packed column GLC.

We chose to form the PFB ethers rather than other derivatives because of the following:

(a) Reproducibility. As indicated in Table 1, the PFBBr reaction was highly reproducible. Yields for the PFB ethers of 29 phenols on 6 replicate derivatizations had a relative standard deviation less than 10% at 1 μg levels. Therefore, this method was sufficiently precise for quantitative purposes. It should be noted that, if the evaporation of reaction products was not carried out properly, the precision on the yields of the PFB ethers of the more volatile phenol, thiophenol as well as the monochlorophenols PFB was lower because of evaporative losses. Precision data for cyanophenol were not determined because of interference from 2-nitrophenol. For the PFBBr reaction with the 2 dinitrophenols, see discussion below.

The effect of reaction time on the yields of derivatives was also examined. No improvement on the yields of the above 29 phenol PFB ethers was noted for the 1, 2, and 3 h reactions relative to the nominal 30 min reaction, indicating that the conditions cited in the experimental section were optimal for maximum yield and reproducible results.

(b) Stability. The PFB ethers of all 32 phenols in hydrocarbon solvents were stable even at room temperature for at least 4 weeks. This was supported by the fact that no substantial changes were observed for the absolute and relative GC responses of all PFB ethers in the same standard solution during such a period of time. Also, no new peaks were observed in the gas chromatogram. On the other hand, the heptafluorobutryates of the more highly halogenated phenols were reported to be unstable and new standards were derivatized daily (12). In this respect, the

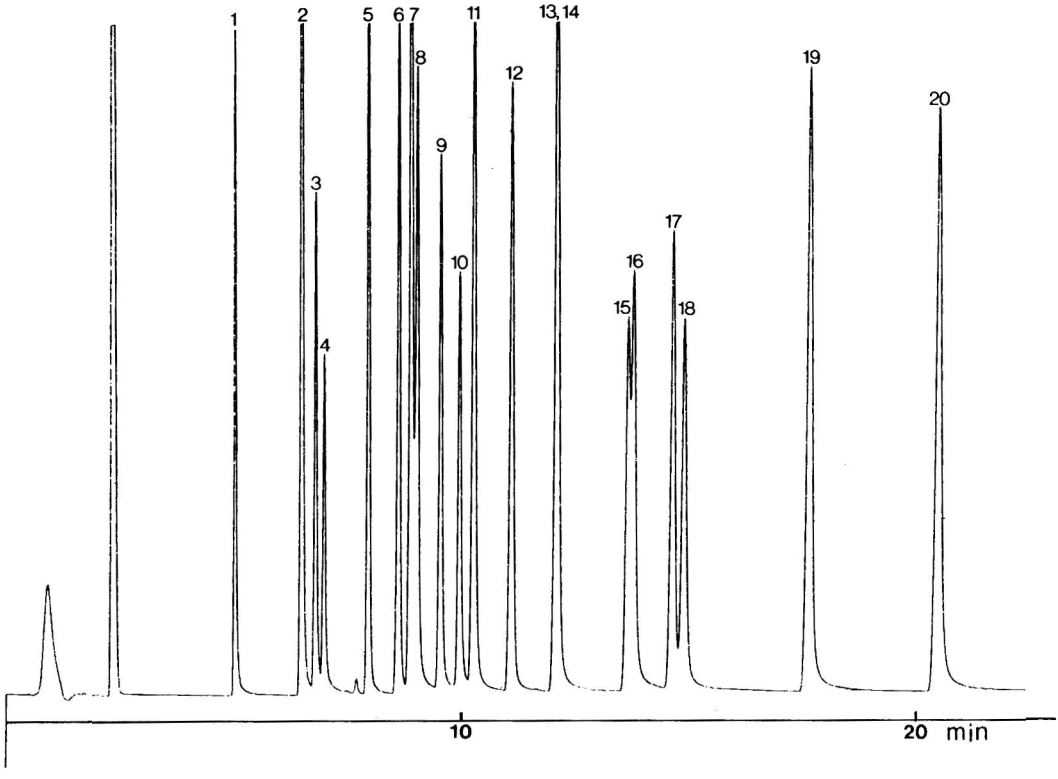

Figure 2. Resolution of PFB ethers for phenols 1-20 on OV-101 FSCC.

PFB derivatives were more desirable than the HFB derivatives.

(c) Sensitivity. The absolute sensitivity limit for all PFB ethers derived from phenols listed in Table 2 was estimated to be between 0.5 to 5 pg injected onto the OV-101 FSCC. This represents an increase of 1000-fold or more in sensitivity as compared to the HPLC-UV detection of underivatized phenols (5). The detection limits of free phenols as determined by injecting standard solutions onto packed GLC columns with FID or ECD were not given in refs 2 and 6, respectively, therefore direct comparison of sensitivity limits cannot be made. In the case of FSCC gas chromatography, it has been stated that determination of free phenols at nanogram and subnanogram levels is difficult on a routine basis because of adsorption problems (7), and therefore the practical sensitivity limits would be drastically reduced.

The ECD response factors of the PFB ethers relative to the PCP derivative are also listed in Table 2. They were all within a factor of 10, suggesting that a nonchlorinated phenol such as 2,4-dimethylphenol can be determined along with a highly chlorinated phenol such as PCP at similarly low levels. Note that, however, these response factors are not corrected for yields of the derivatives.

(d) Versatility. The PFB derivatization works equally well for the analysis of at least 10 common acid herbicides (15). In addition, the same technique was also successfully applied to the analysis of several carbamates (16) and the confirmation of organophosphorus pesticides (3, 4).

Derivatization of Dinitrophenols

The 2 dinitrophenols do not react with PFBBr in the same way as the other 30 phenols in Table 1. After 30 min reaction at 60°C, the yields for the 2 PFB ethers were extremely low. The reaction was then monitored at regular intervals from 1 h up to 6 h. The results of these experiments indicated that the yields of the 2 PFB ethers increased with time up to about 5 h and then leveled off. The ECD response of the products were considerably lower (~50 times) than other phenol derivatives, indicating either that the ECD is insensitive to these PFB ethers or the yield is low. These 2 dinitro derivatives

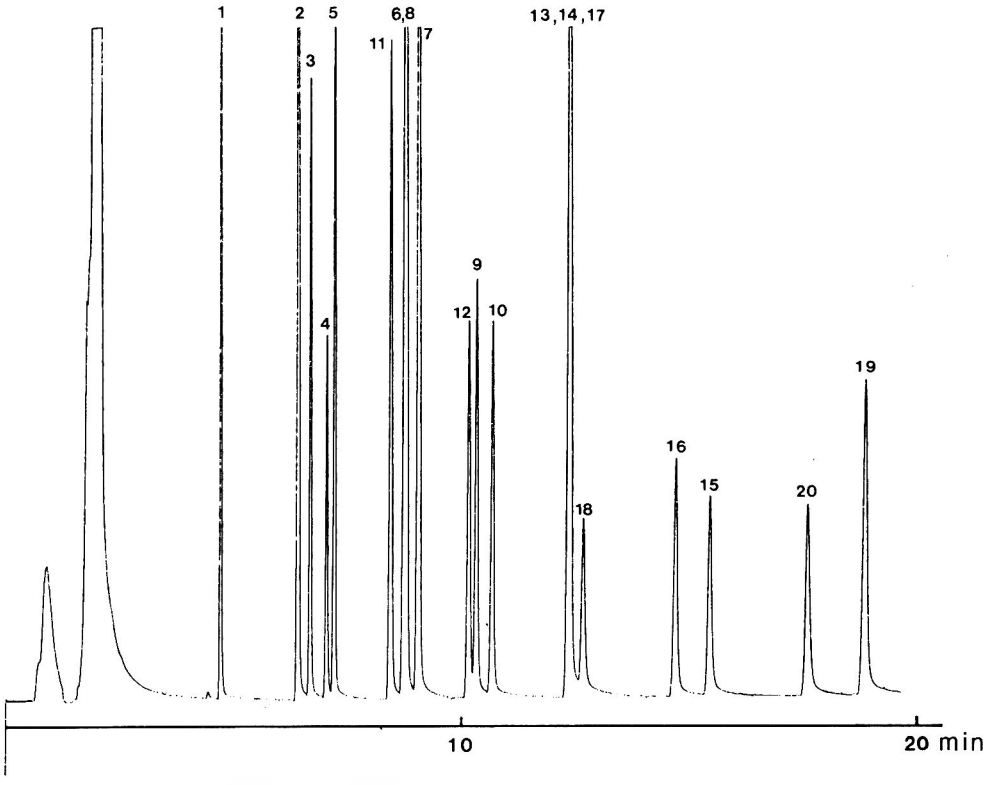

Figure 3. Resolution of PFB ethers for phenols 1-20 on Carbowax 20M FSCC.

need to be synthesized and isolated before these questions can be unequivocally answered.

Resolution of Phenol PFB Ethers on GLC Columns

The separation of phenol PFB ethers was studied isothermally with packed columns. For comparison, FSCC chromatography was also investigated using splitless injection and 2-ramp temperature programming as detailed in the experimental section.

The retention times and relative retention times of the 32 phenol PFB ethers on 6 columns, namely (a) 3% OV-1, (b) 3% OV-17, (c) Ultrabond 20M, (d) OV-101 FSCC, (e) SE-54 FSCC, and (f) Carbowax 20M FSCC are listed in Table 1. These columns were selected because of their wide range of stationary phase polarity as well as their popularity and commercial availability.

In the present case of phenol PFB ethers, 2 peaks with a difference in retention times of 0.2 min on an FSCC would normally have baseline resolution, while a partial splitting of peaks could be obtained with RT difference of about 0.1 min. Therefore, the retention time data listed in Table 1 enable us to determine whether or not the pair of derivatives in question is unresolved, partially, or totally resolved on a FSCC.

To facilitate the discussion, the phenols are grouped according to the fractionation pattern of their PFB ethers from a mini-silica gel column as described in the experimental section. Since chlorophenols are important as a class by themselves, all 19 chlorophenols are also discussed as a group separately.

Mini-Silica Gel Column Fractions.—Fraction 1 contains 26 phenols (Nos. 1-26, Table 1). Separation was achieved on the OV-101 FSCC for all derivatives except for the following pairs: 2-chlorophenol and 2,4-dimethylphenol (Nos. 2 and 21, both are U.S. EPA priority pollutants) as well as 2,3,5- and 2,4,5-trichlorophenols (Nos. 13 and 14). In addition to the above pairs, the SE-54 column did not resolve the 2,4- and 3,5-dichlorophenol derivatives. Under the conditions used, the Carbowax 20M FSCC was less efficient and the unresolved pairs included derivatives of 4-chlorophenol and 2-chloro-5-methylphenol (Nos. 4 and 23), the 2,5- and 3,5-dichlorophenols (Nos. 6 and 8), 2,4-dichlorophenol and 2-

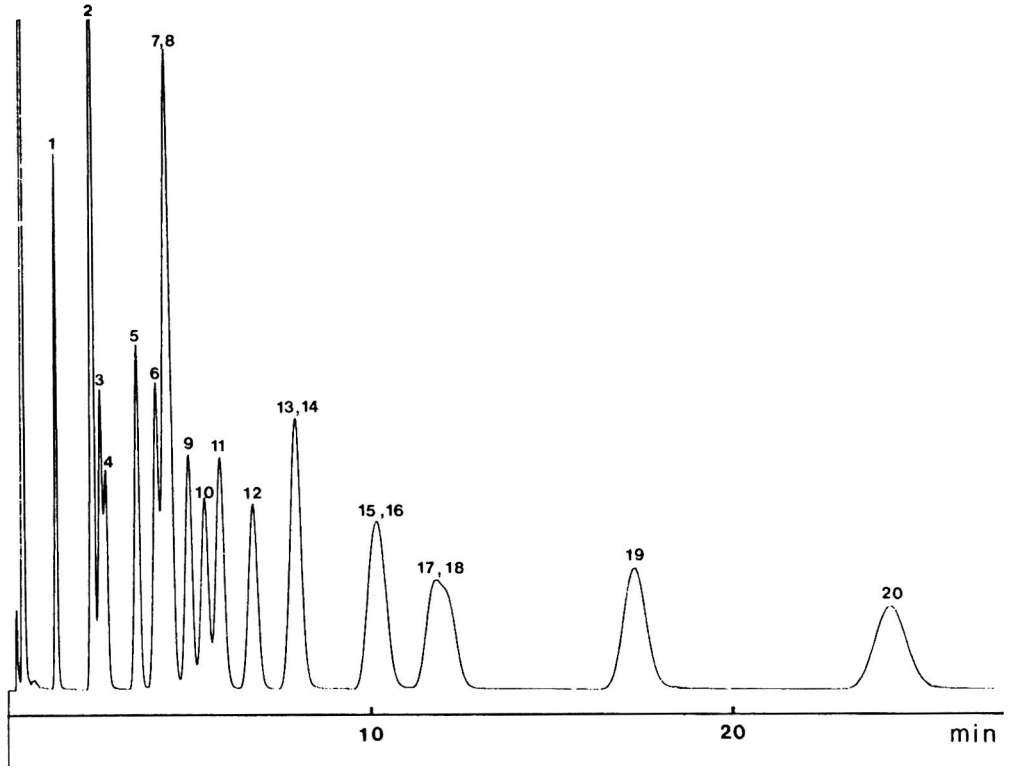

Figure 4. Resolution of PFB ethers for phenols Nos. 1-20 on 3% OV-1 column.

chloro-4-tert-butylphenol (Nos. 7 and 25), as well as 2,4,5-trichlorophenol, 2,3,5-trichlorophenol, and 2,3,5,6-tetrachlorophenol (Nos. 13, 14, and 17). However, the Carbowax FSCC resolved the 2-chlorophenol and 2,4-dimethylphenol derivatives. It was therefore obvious that the most efficient column for these phenol derivatives was the nonpolar OV-101 FSCC. In summary, except for the derivatives of 2,3,5- and 2,4,5-trichlorophenols, all phenol PFB ethers in fraction 1 could be resolved by a combination of the OV-101 and Carbowax 20M FSCC.

Separation of a complicated mixture such as the 26 PFB ethers in fraction 1 on packed columns is, on the whole, less efficient than that obtained from FSCC. However, packed columns are still useful for less complicated mixtures and the retention time data in Table 1 enable the reader to choose a suitable column for the phenols of particular interest. The data also provide information on possible interference when other phenols in the same table are also present in the mixture.

Fraction 2 contains 4 phenol derivatives (Nos. 27 to 30). All 6 columns listed in Table 1 resolved the mono-nitrophenol derivatives easily. However, resolution of the 4-cyanophenol and 2-nitrophenol derivatives could be obtained only on the Ultrabond packed column or the Carbowax 20M FSCC. Because of the polarity of the derivatives in fraction 2, their retention times on the 2 polar columns (Ultrabond and Carbowax 20M) was much longer than those obtained on the other 4 nonpolar or slightly polar columns.

Fraction 3 contains the 2 dinitrophenol derivatives (Nos. 31 and 32, both are U.S. EPA priority pollutants). These derivatives were the most polar ones among the phenols studied and they were eluted from the silica gel column in the third fraction by a more polar eluant. This phenomenon is advantageous in the multiresidue analysis of these phenols because the 2 dinitrophenol ethers have long retention times on most GLC columns. Thus more efficient GLC conditions (e.g., higher column temperature, shorter column, different stationary phase) can be applied to the packed column GLC analysis of the dinitrophenol derivatives. Such conditions

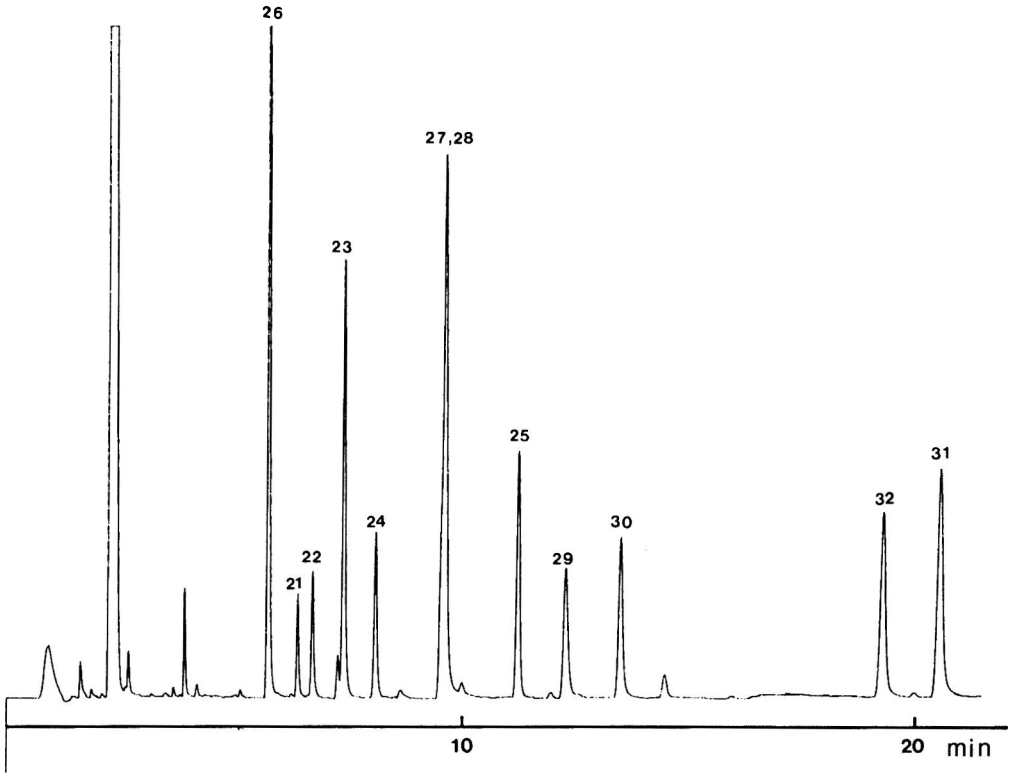

Figure 5. Resolution of PFB ethers for phenols Nos. 21-32 on OV-101 FSCC.

are usually not efficient for resolution of the more volatile phenol derivatives. The dinitro derivatives were resolved on the nonpolar and slightly polar columns. However, the same derivatives did not chromatograph on either the Ultrabond 20M column or the Carbowax 20M FSCC under the conditions used in this work.

GLC Characteristics of 19 Chlorophenol Derivatives.—For the 19 chlorophenols, the retention times of the isomeric derivatives on the nonpolar 3% OV-1 column and OV-101 FSCC show a marked dependence on the relative positions of substitution in the following manner: (i) Isomers with adjacent chlorine atoms have longer retention times than isomers with nonadjacent chlorine atoms; (ii) isomers with a greater number of adjacent chlorine atoms have longer retention times; (iii) for isomers with substitution in the ortho, meta, and para positions, retention times increase in the order o < m < p. These rules are well illustrated by the order of elution of the 4 series of isomeric chlorophenol PFB ethers as depicted in Figure 1. The order of elution for those ethers on the slightly polar OV-17 and SE-54 columns as well as the polar Carbowax columns is generally the same with only a few exceptions. However, in the case of the Carbowax columns, the order of elution dichloro- < trichloro- < tetrachloro- < pentachloro- does not hold. For example, the PCP derivative elutes earlier than that of 2,3,4,5-tetrachlorophenol, and PFB ethers of both 2,3,5,6- and 2,3,4,6-tetrachlorophenols elute earlier than those of 3,4,5- and 2,3,4-trichlorophenol, etc. Because of this change in elution pattern, the Carbowax columns are useful for confirmation purposes.

Typical chromatograms for all 19 chlorophenol PFB ethers on the OV-101 and Carbowax 20M FSCC are shown in Figures 2 and 3, respectively. For comparison, the separation of these same derivatives on a 3% OV-1 packed column is shown in Figure 4.

U.S. EPA Priority Phenols and Others.—Separation of PFB ethers for phenols 21-32 on the OV-101 FSCC is illustrated in Figure 5. For the PFB ethers of the 11 U.S. EPA priority phenols, the most efficient column was the 3% OV-17 packed column and a typical chromatogram is shown in Figure 6. For this particular group of phenols,

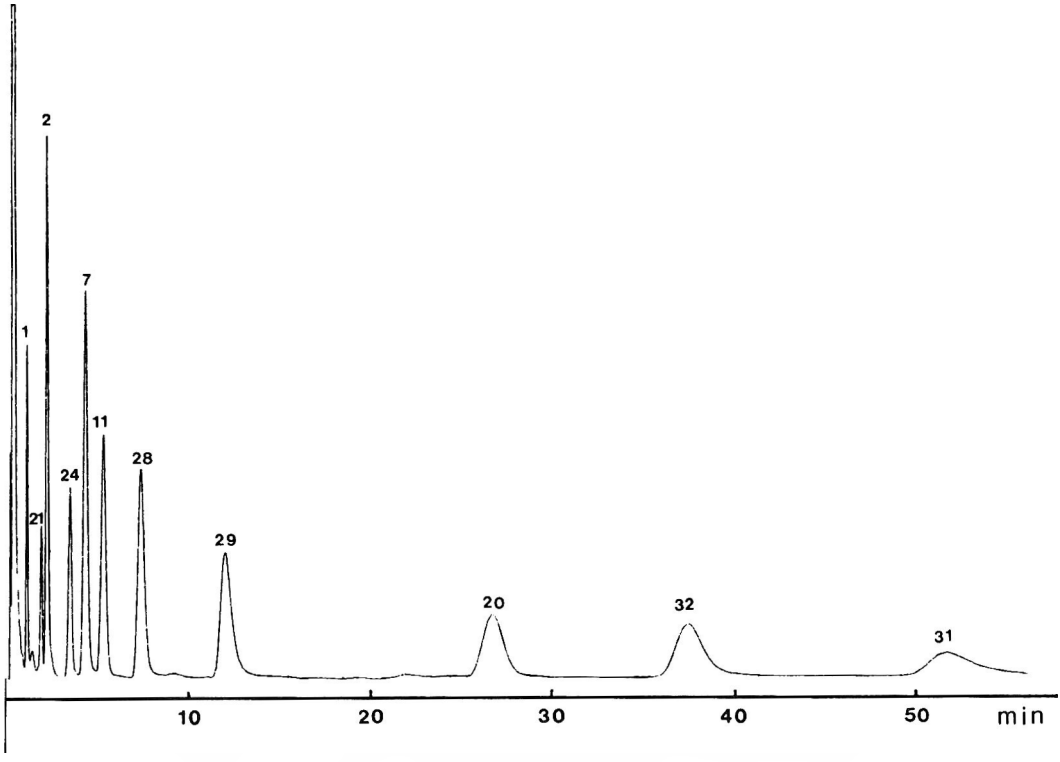

Figure 6. Resolution of 11 U.S. EPA phenol-PFB ethers on 3% OV-17 column.

neither one of the 3 FSCC listed could completely resolve the 11 derivatives for situations already discussed.

Acknowledgments

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COLOR ADDITIVES

High Performance Liquid Chromatographic and Colorimetric Determination of Synthetic Dyes in Gelatin-Containing Sweets, Following Polyamide Adsorption and Ion-Pair Extraction with Tri-*n*-octylamine

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Dyes are determined in gelatin-containing sweets. The gelatin must be eliminated first because it interferes with the normal ion-pair extraction of dyes with tri-n-octylamine to chloroform. Techniques such as precipitation of gelatin with organic solvents, and acid and enzymatic digestion of gelatin are shown to be unsuccessful because the remaining gelatin still influences the extraction scheme. Positive results are obtained when dyes are adsorbed on polyamide, gelatin is washed away, and dyes are desorbed with a methanol-ammonia mixture. Dyes are identified by thin layer chromatography and high performance liquid chromatography (HPLC), and quantitated by HPLC or colorimetry.

Synthetic dyes have been isolated in the past by various techniques which we reviewed in previous papers (1, 2). From these procedures, we selected ion-pair extraction with tri-*n*-octylamine (TnOA) and in this paper we demonstrate its applicability in the analysis of sweets. When sweets consist mainly of sugar, there is no problem in extracting the dye directly with TnOA after simply dissolving the food in water. However, there are problems in analyzing sweets having a high gelatin content. Indeed, preliminary experiments indicated that no extraction at all occurred when dyes were extracted with TnOA in the presence of gelatin (a denatured protein). Hoogewijs and others (3, 4) working with ion-pair extraction of basic drugs from human plasma with *n*-octyl sulfate as the counter-ion, showed formation of a ternary complex between the solute, the counter-ion, and the proteins of the plasma, which interfered in the extraction of the solute. A similar phenomenon may be present in extraction of acid dyes with an amine as the counter-ion. It is also a well established fact that proteins adsorb drugs in biological fluids; analogously, dyes are bound to gelatin. This binding could also explain the low extraction yields obtained with TnOA because the binding forces might be stronger than the attraction forces involved in ion-pair formation. Extractions from 0.1M HCl to chloroform also have a very low extraction yield. This means that the inhibited extraction is due mostly to the binding of the dyes to gelatin. Hoogewijs and Massart resolved their problem by precipitating the proteins with an organic solvent, e.g., acetonitrile. We also attempted to eliminate the protein prior to ion-pair extraction; our methods are discussed in this paper.

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Experimental

Apparatus and Reagents

(a) *Spectrophotometer.*—Perkin Elmer Hitachi 200.

(b) *pH meter.*—Orion Ionanalyser 601 and combined glass electrode.

(c) *Liquid chromatograph.*—Varian LC 5060 equipped with Valco loop injector (100 μL loop); Varian 254 nm detector, and Varian Varichrom® variable wavelength detector. Chromatograms were usually monitored at 254 nm with the fixed wavelength detector; for other wavelengths the variable detector was used. RP18 Lichrosorb column, 10 μm octadecylsilica, 250 mm × 4.6 mm id (Merck). Peak areas were calculated with a Varian Vista 401 integrator.

(d) *Mobile phases for HPLC.*—5 mL tetrabutylammonium hydroxide (25% in methanol, Fluka) was diluted to 1 L with mixtures of methanol-phosphate buffer (pH 7.00 ± 0.05, ionic strength 0.1): phase A = 30 + 70, phase B = 60 + 40.

Mobile phases were prepared by mixing appropriate volumes of phases A and B. Flow rate was 60 or 120 mL/h. Chromatographic system was described in previous papers (1, 2).

(e) *TLC system.*—Cellulose precoated plates (Merck, GFR) 20 × 20 cm, 0.1 mm thick. Mobile phase: ethyl acetate-*n*-propanol-ammonia-water (35 + 35 + 20 + 20). TLC system was described earlier (1, 2).

pH	H_3PO_4, 1M (mL)	$NaH_2PO_4 \cdot H_2O$ (g)	$Na_2HPO_4 \cdot 2H_2O$ (g)	$Na_3PO_4 \cdot 12H_2O$ (g)
3.0	32.0	27.598	—	—
4.0	3.2	27.598	—	—
5.0	—	26.580	0.427	—
5.5	—	24.650	1.260	—
7.0	—	5.768	9.380	—
10.0	—	—	11.391	0.855

(f) *Dyes.*—Purchased from P. Entrop (Machelen, Belgium) and used as received.

(g) *Chemicals.*—(1) Tri-*n*-octylamine (Aldrich Europe) used as received.

(2) Crystallized pepsin (Armour Pharmaceutical Co., Eastbourne, UK).

(3) Subtilisin Carlsberg (crystallized from *Bacillus subtilis*, Sigma, München, GFR).

(4) Polyamide for column chromatography (Woelm, Eschwege, GFR).

(5) All other reagents and solvents were prepared from analytical grade products (Merck).

(h) *Buffers.*—Phosphate buffers of ionic strength 0.1 were prepared by mixing amounts shown and diluting mixtures to 1 L with double distilled water. If necessary, the pH was adjusted with 1M H_3PO_4 or 1M NaOH.

Adsorption of Dyes on Polyamide

In a stoppered 30 mL centrifuge tube, 10 mL of a dye solution containing 1 mg/100 mL of an adequate phosphate buffer was added to 10 mL of the same buffer as well as 250, 500 or 1000 mg polyamide powder. The tube was shaken for 15 min and centrifuged, and the absorbance of the supernatant liquid was measured vs dye standards prepared in the appropriate buffer solution. Triplicate adsorption determinations were made and the results were averaged.

Desorption of Dyes from Polyamide

In a stoppered 30 mL centrifuge tube, the dyes adsorbed on 1000 mg polyamide were desorbed with 15 mL methanol-ammonia (95 + 5) by shaking 15 min, centrifuging, and removing the supernatant liquid by pipet. The procedure was repeated twice and the combined extracts were collected and diluted to a total volume of 50 mL with methanol-ammonia (95 + 5). The absorbance of this solution was measured by colorimetry vs dye standards prepared in methanol-ammonia. Triplicate desorption determinations were made and results were averaged.

Procedure for Commercial Samples

Samples (1 to 3 sweets, 3–10 g) were weighed and dissolved in 20 mL warm pH 4.0 phosphate buffer. The solution was added to 1 g polyamide in a stoppered 30 mL centrifuge tube. After 15 min shaking, the tube was centrifuged, the supernatant liquid was removed by pipet, and the polyamide was washed 3 times (or more if necessary) with 10 mL portions of hot water by shaking the tube 5 min and centrifuging. The colorants were desorbed by shaking the polyamide 3 times with 15 mL methanol–ammonia (95 + 5). The combined extracts were evaporated to dryness at 40°C, the residue was redissolved in 10 mL pH 5.5 phosphate buffer, and the dyes were extracted with 5 mL 0.1M TnOA in chloroform. Dyes were identified by thin layer chromatography (TLC) of 10 μL of this phase. They were also identified and determined by high performance liquid chromatography (HPLC) of the perchlorate phase obtained by extracting 3 mL of the TnOA phase in chloroform with 3 mL 0.1M sodium perchlorate. For samples containing only one dye, the dye could be determined by colorimetry after diluting the methanol–ammonia phase to 50 mL.

Results and Discussion

Precipitation of Gelatin

Treatment of colored gelatin solutions with methanol, ethanol, acetonitrile, and acetone all resulted in incomplete precipitation of gelatin, and consequently the ion-pair extraction was still inhibited.

Hydrolysis of Gelatin

Acid digestion was too drastic for use in analyzing commercial sweets because of carbonization of sugar. More gentle hydrolysis techniques (enzymatic digestion), used by Boley et al. (5, 6) in food analysis and by Osselton et al. (7, 8) in the analysis of drugs in tissues, were tried. A normal extraction is obtained but TLC spots

showed significant tailing, probably due to the presence of amino acids which co-extract.

Polyamide Adsorption and Desorption

Another means of separating dye from the gelatin could consist of adsorbing the dye and then washing the gelatin away. Such a procedure was proposed by Lehmann et al. (9) who solubilized the dye, adsorbed it on polyamide in acetic acid medium, transferred the slurry to a glass chromatographic column, repeatedly washed the column with a variety of solvents, and finally desorbed the dye with methanol-ammonia or methanol-sodium hydroxide. However, they gave no recovery data, and, furthermore, the procedure is very tedious and requires several hours. Nevertheless, their method yields useful extracts in which extracted dyes are identified successfully.

This method was also used by Gilhooley and coworkers (10) as an extraction technique with some changes (11), e.g., the replacement of polyamide by a silica gel-cellulose mixture. Later the polyamide method was also used by Boley et al. (5, 6), Sabir et al. (12), and Chudy and coworkers (13). All these techniques use a chromatographic column. To increase its speed, we changed the original methodology and used it only to eliminate the interfering gelatin. A major change consisted of performing the adsorption and desorption in a stoppered centrifuge tube with a smaller amount of polyamide than in the column techniques. At first adsorption yields were determined for 5 test dyes at various pH values (3–5) and using various amounts of polyamide (0.25–1 g).

The 5 test dyes (Tartrazine, Amaranth, Erythrosine, Indigotine, and Brilliant Green) are all adsorbed quantitatively by the polyamide even with the smallest amount tested (0.25 g). Since Lehmann et al. (9) stated that adsorption should take place in an acetic acid medium, the influence of pH on the adsorption yield was also investigated. The test dyes were extracted quantitatively, and we concluded that pH is not critical in the pH interval investigated. The adsorption of the other dyes was investigated only at pH 4.0 and using 1 g polyamide. Patent Blue V, the most poorly adsorbed dye, still shows an adsorption yield of 97.4% which is quite acceptable. The desorption of dyes was carried out with methanol-ammonia (95 + 5) according to Lehmann et al. (9). We also applied this mixture with success in the desorption of Tartrazine from a rice milk matrix (14), with quantitative determination. This mixture was preferred to methanol-sodium hydroxide because of the volatility of its components, which is quite important considering the evaporation step following desorption (see below). Yields following adsorption + desorption are given in Table 1, column A. Desorption is complete after the first desorption. It is repeated, however, to also collect the dye, which is already dissolved in the methanol-ammonia phase but is still kept between the polyamide particles. The data in Table 1, column A, indicate overall yields (adsorption + desorption) which are equal to the desorption yields except for Patent Blue V which was adsorbed for only 97.4% recovery. The adsorption + desorption yields range from 92 to 103%, which is acceptable.

Recovery Through Entire Extraction Scheme

Table 1, column B, shows the recovery of the dyes, previously adsorbed on and desorbed from polyamide and extracted with TnOA. Recoveries range from 90 to 102% which are quite similar to the previous step, and indicate that the TnOA extraction is nearly quantitative. In the

Table 1. Recovery of dyes in various steps of the extraction procedure

Dye	A, adsorption + desorption		B, A + extraction with TnOA		C, B + back-extraction with ClO_4^-	
Tartrazine	97.9 ± 0.3	(97.7)[a]	97.9 ± 0.5	(97.7)	96.2 ± 0.8	(96.4)
Amaranth	100.2 ± 0.3	(100.0)	99.8 ± 0.7	(99.6)	99.8 ± 1.2	(99.4)
Erythrosine	95.3 ± 0.3	(95.5)	93.5 ± 0.7	(93.4)	5.9 ± 0.9	(5.2)
Indigotine	92.7 ± 0.6	(92.1)	90.1 ± 0.8	(89.8)	88.3 ± 1.3	(88.0)
Brilliant Green	102.8 ± 1.0	(102.6)	102.0 ± 1.0	(101.6)	65.8 ± 1.4	(64.9)
Quinoline Yellow	91.8 ± 0.3	(92.0)	91.6 ± 0.9	(91.6)	90.5 ± 1.2	(90.6)
Sunset Yellow	101.8 ± 0.3	(101.5)	101.5 ± 0.9	(101.0)	100.1 ± 1.4	(100.1)
Azorubine	96.8 ± 0.3	(97.1)	96.6 ± 0.9	(96.2)	92.0 ± 1.5	(92.2)
Cochenille Red	97.7 ± 0.3	(97.7)	97.7 ± 1.0	(97.3)	97.4 ± 1.5	(97.5)
Patent Blue V	92.5 ± 0.5	(92.7)	91.8 ± 1.1	(91.6)	84.5 ± 1.8	(84.4)
Brilliant Black BN	96.3 ± 0.3	(96.5)	96.3 ± 0.6	(96.2)	90.1 ± 1.2	(90.0)

[a] Values in parentheses show recovery from 5% gelatin solution, instead of water solution.

back-extraction with perchlorate (Table 1, column C), losses range from negligible (Tartrazine, Amaranth, Quinoline Yellow, Sunset Yellow, Cochenille Red) and small (Indigotine, Azorubine, Patent Blue, Brilliant Black) to large (Brilliant Green) and very large (Erythrosine). Consequently, for Erythrosine and Brilliant Green, it would be preferable to measure the chloroform-TnOA phase. The values between parentheses in Table 1 show recovery of dyes from initial solutions containing 5% gelatin. From these data it may be concluded that gelatin does not influence extraction. Recovery was also determined when Tartrazine, Amaranth, and Patent Blue V were added to blank gelatin sweets. The following yields were obtained for steps A, B, and C, respectively: Tartrazine, 97.6 ± 0.5, 97.5 ± 0.4, 95.9 ± 0.9; Amaranth, 100.0 ± 0.4, 99.9 ± 0.6, 99.7 ± 0.9; Patent Blue V, 92.0 ± 0.6, 91.6 ± 1.0, 85.2 ± 1.1. These values are equivalent to those obtained from aqueous or 5% gelatin solutions, which means that the procedure should be applicable to colored commercial gelatin-containing sweets.

For 3 dyes (Indigotine, Erythrosine, and Brilliant Green), the procedure should be carried out as fast as possible to prevent discoloration which takes place especially in alkaline medium (1, 15).

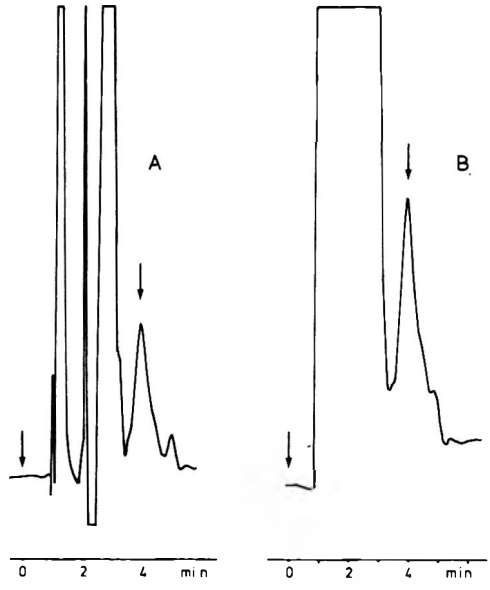

Figure 1. Identification of Erythrosine in sweet sample 6 by direct injection of the chloroform-TnOA extract. Mobile phase, 100% B; flow rate, 120 mL/h; detection, 254 nm; volume injected, 100 μL; 0.02 AUFS. A, standard Erythrosine 0.05 mg/100 mL chloroform; B, sample extract containing 0.098 mg/100 mL.

Analysis of Commercial Samples

A sufficient number of sweets, depending on their size and color content, were analyzed by using the polyamide methodology. The gelatin, which is not adsorbed on the polyamide, was washed away with hot water, and the dyes were desorbed with methanol-ammonia. This solution was evaporated to dryness, the residue was redissolved in a phosphate buffer, and the dyes were extracted with TnOA in chloroform. This latter extraction is still necessary because direct chromatography of the methanol-ammonia phase is not successful due to the presence of disturbances, possibly emulsifiers, which interfere in TLC of the dyes. Direct injection of the methanol-ammonia phase into the HPLC system is also impossible because the retention of dyes is drastically disturbed when dyes are chromatographed in such a solution. The changes introduced to the Lehmann method by Gilhooley et al. (10, 11) consisted of replacing the adsorbing material or the elution solvent to obtain a purer extract, and did not intend to increase the speed of the method. Boley et al. (6) replaced the second treatment with polyamide by an extraction and a back-extraction just as we do. However, in their method, the extraction precedes the polyamide treatment and in our method it follows. Our method is faster than that of Boley et al. because we replaced column methodology by a shaking and centrifugation technique which is easier to carry out. The recoveries over the whole method are generally similar or a few percent higher with our method. For Indigotine, our method is much better, but for Erythrosine, Boley's method gives a much higher yield. As can be seen, in Table 1, columns B and C, the losses of Erythrosine occur in the back-extraction. The identity of the dyes is determined by TLC of the chloroform extract, and confirmed by HPLC of the perchlorate extract by monitoring the eluate at a specific wavelength.

Figure 1 shows that it is possible to directly inject the TnOA chloroform extract in the reverse phase system, with however, quite important solvent peaks. These peaks pass unobserved when the eluate is monitored at the wavelength of maximal absorption (in the visible field of the spectrum) of the compound (Figure 2). This approach, i.e., direct injection of chloroform extract, was necessary only for Erythrosine because of its low extraction yield with perchlorate (1) (see Table 1). Figures 1 and 2 show the identi-

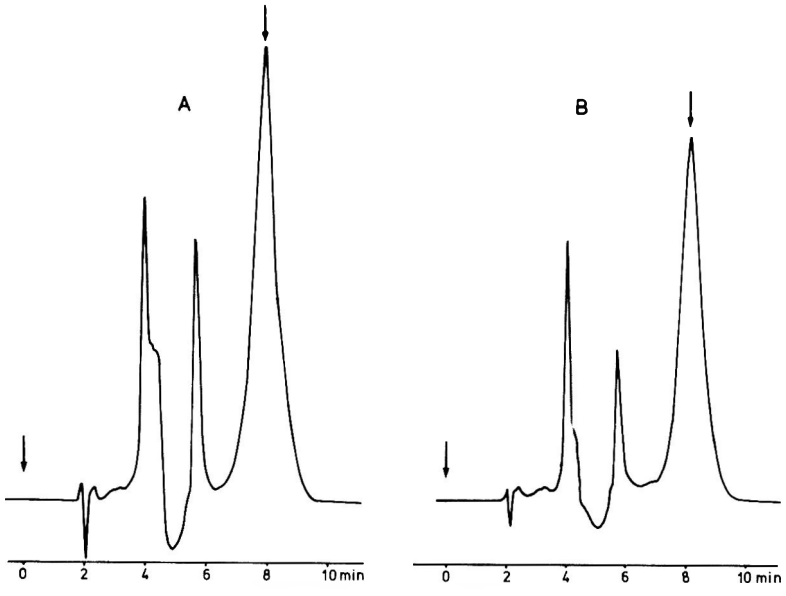

Figure 2. Identification of Erythrosine in sweet sample 5 by direct injection of the chloroform-TnOA extract. Mobile phase, 100% B; flow rate, 60 mL/h; detection, 526 nm; volume injected, 100 μL; 0.1 AUFS. A, standard Erythrosine 0.2 mg/100 mL chloroform; B, sample extract containing 0.148 mg/100 mL.

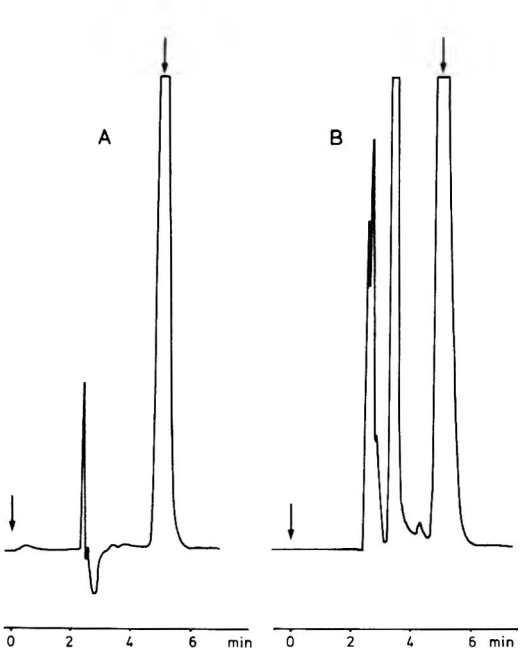

Figure 3. Identification of Tartrazine in sweet sample 2 by injection of the perchlorate extract. Mobile phase, 30% A-70% B; flow rate, 60 mL/h; detection, 254 nm; volume injected, 100 μL; 0.08 AUFS. A, 10 ppm standard Tartrazine in water; B, sample extract containing 2.63 mg/100 mL.

Table 2. Determination of dyes (mg/kg) in different commercial sweets

Sample	Dye	No. pieces taken for anal. 1	2	3
1	Tartrazine	50.5	47.4	70.7
	Erythrosine	3.6	3.6	4.6
2	Tartrazine	5.3	5.6	8.2
3	Tartrazine	0.7	0.8	0.9
	Patent Blue V	0.4	0.7	0.8
4	Erythrosine	37.6	32.7	
5	Erythrosine	38.9	40.0	
6	Erythrosine	8.4	9.4	9.6
7	Erythrosine	1.4	1.7	
8	Indigotine	31.4		
9	Tartrazine	21.3	22.3	21.0
	Indigotine	3.5	3.6	3.6
10	Erythrosine	<0.1	<0.1	<0.1
11	Tartrazine	41.1	40.4	46.1
12	Tartrazine	61.5	59.5	57.6
13	Tartrazine	35.1	35.0	34.9
14	Tartrazine	1.0	0.9	0.9
	Patent Blue V	2.1	2.0	1.9

fication of Erythrosine; Figure 3 shows the identification of Tartrazine in a sweet. Single dyes were determined by colorimetry of the methanol-ammonia phase. When more than one dye was present, dyes were determined by HPLC. In calculating the analytical result, the

adsorption and desorption yield was taken into account.

Results on commercial samples are given in Table 2. The values given indicate the dye found in 1, 2, or 3 sweets. If possible, a triplicate determination was carried out for each kind of sweet which gives an idea of the between-samples variation. The within-sample variation coefficient (= analytical variation) of the method itself is 2–5%. The detection limit depends on several factors: the extinction coefficient of the dye and the measurement technique employed (HPLC or colorimetry). If colorimetry is chosen, 1 or 5 cm cells may be used; in HPLC, the detector range may descend to 0.005 AUFS. With the method described above, detection limits range from 0.1 to 0.5 mg/kg.

Acknowledgments

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TECHNICAL COMMUNICATIONS

Report on Disinfectants

RETO ENGLER
Environmental Protection Agency, Registration Division, Washington, DC 20460

Tubercocidal Test: Associate Referee J. M. Ascenzi reports that studies to improve the reliability and reproducibility of the tubercocidal test are still ongoing. However, no test method has developed which is ready for collaborative testing.

Use-Dilution Test: Associate Referee G. Walter reports that activities in his area have been held in abeyance. However, means of improving the test will be considered. The AOAC membership is encouraged to make suggestions, based on laboratory experience.

Sporicidal Tests: T. Wendt, Surgikos, has been studying means to improve sporicidal testing. He should be appointed Associate Referee for those tests.

Bacteriostatic Activity of Textile Additives: L. B. Arnold and L. Smith are Co-Associate Referees for testing bacteriostatic activity of textile additives. A quantitative assay for evaluating textile additives is under development.

Virucidal Tests: Associate Referee C. McDuff intends to pursue research to determine whether drying of viruses onto hard surfaces is a desirable step in the virucidal tests. Preliminary studies will be undertaken to determine the susceptibility to disinfectant of several viruses in the dry and liquid state.

It is recommended that all studies continue.

This report of the General Referee was presented at the 96th Annual International Meeting of the AOAC, Oct. 25–28, 1982, at Washington, DC.

The recommendation of the General Referee was approved by Committee F and was accepted by the Association. See the report of the Committee, *J. Assoc. Off. Anal. Chem.* (1983) **66**, March issue.

Direct Testing of Gelatin Hydrolysis in Rapid Perfringens Medium

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Rapid perfringens medium (RPM) was previously shown to be effective for detection of low numbers of *Clostridium perfringens* in foods. The detection system of RPM is based on a stormy fermentation of litmus milk. RPM also contains 6% gelatin, and the present work was performed to determine if gelatin hydrolysis can be tested directly in this medium. Twenty-three strains of *C. perfringens* and related species were inoculated into tubes of RPM and lactose gelatin. All strains were positive for stormy fermentation on RPM after 24 h incubation at 46°C. Seventeen exhibited gelatin hydrolysis on both RPM and lactose gelatin. Tubes of RPM inoculated with slurries of food samples spiked with each of 11 strains of *C. perfringens* were positive for stormy fermentation and gelatin liquefaction. Most *C. perfringens* hydrolyze gelatin, so this test augments stormy fermentation in RPM as an additional indicator for the presence of this pathogen.

In 1978, Erickson and Deibel (1) described and evaluated a new medium, called rapid perfringens medium (RPM), for the detection and enumeration of *Clostridium perfringens* in foods. RPM is a liquid medium with a litmus milk base and is prepared in tubes. Selectivity is provided by the antibiotics polymyxin-B sulfate and neomycin sulfate, coupled with an incubation temperature of 46–48°C. Detection of *C. perfringens* is based on production of a stormy fermentation reaction within 24 h. However, stormy fermentation is only a presumptive test, and isolates should be confirmed by the additional tests listed

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by Erickson and Deibel (1). Using RPM, these authors found confirmed *C. perfringens* in 71% of several hundred naturally contaminated food samples, compared with 22% with sulfite-polymyxin-sulfadiazine agar (2) and 14% with tryptose-sulfite-cycloserine agar (3).

Many investigators (1, 4–7) have shown that gelatin hydrolysis is a useful test for distinguishing *C. perfringens* and other closely related clostridia. Some heat-resistant strains, however, do not hydrolyze gelatin (8). RPM contains 6% gelatin, originally added to stabilize the medium during autoclaving, and it seemed possible that a test for gelatin hydrolysis could be performed directly in RPM. This possibility was tested in the present study.

Experimental

Cultures and Media

The cultures of clostridia used in this study were provided by Stanley M. Harmon, Division of Microbiology, Food and Drug Administration, Washington, DC 20204. Stock spore suspensions were stored in sterile milk at 4°C. Working cultures were maintained in buffered trypticase-peptone-glucose-yeast extract medium (TPGY). This medium has the same formulation as TPGY medium described in the *Bacteriological Analytical Manual* (BAM) (9), except that 0.5% disodium phosphate is included and the pH is adjusted to 7.3 before sterilization.

Gelatin Liquefaction

Pure cultures of 17 strains of *C. perfringens*, 2 strains of *C. paraperfringens*, 2 strains of *C. sardiniensis*, 1 strain of *C. barati*, and 1 strain of *C. absonum* were studied to determine whether gelatin hydrolysis could be shown directly in RPM. Duplicate tubes of RPM and lactose gelatin (9) were inoculated with 0.1 mL of 24 h cultures of each strain grown in buffered TPGY medium. The RPM was incubated for 24 h at 46–48°C and observed for typical stormy fermentation reactions. Tubes of lactose gelatin were incubated for 24 h at 35°C. All tubes of both media including uninoculated controls were then refrigerated for 2 h at 4°C and tested for gelatin liquefaction by inverting the tubes. Liquefaction was indicated if the medium flowed freely down the walls of the inoculated tubes.

Inoculated Products

Fifty g samples of oregano, black pepper, paprika, liver powder, and thyroid powder were inoculated with 1 mL of 24 h cultures of typical *C. perfringens*. Eleven strains of *C. perfringens*, all capable of gelatin hydrolysis, were employed for a total of 55 determinations. Each inoculated sample was blended with 450 mL peptone H_2O (9), and 1 mL of each slurry was placed in a tube of RPM. The tubes were incubated 24 h at 46–48°C and observed for stormy fermentation and gelatin hydrolysis.

Results and Discussion

Stormy fermentation and gelatin liquefaction reactions observed with the clostridial strains are shown in Table 1. All cultures exhibited stormy fermentation reactions in RPM. Seventeen of the cultures exhibited gelatin hydrolysis on lactose gelatin medium. These same 17 cultures tested were known strains of *C. perfringens* and all except 3 were positive for gelatin liquefaction on both media. Two gelatin-negative strains of *C. perfringens* (A-86, IU-3344) had originally been capable of gelatin hydrolysis; however, these reactions apparently changed after several years of maintenance of these cultures in the FDA collection.

The other species of clostridia included in this study were selected because they produce stormy fermentation and can be confused with *C. perfringens*. All 6 strains tested showed negative gelatin reactions within 24 h. However, 2 strains (*C. absonum* ATCC 27636 and *C. sardiniensis* FD-36 and FD-0356) did show gelatin liquefaction in both media after 5 days.

All 55 tubes of RPM which had been inoculated with samples of oregano, black pepper, paprika, and liver powder that had been spiked with typical *C. perfringens* exhibited both stormy fermentation and gelatin hydrolysis in 24 h.

The results of these studies show that RPM medium can be used directly to determine gelatin hydrolysis. This test augments stormy fermentation of litmus milk as an additional indicator for the presence of *C. perfringens*.

Analysts familiar with *C. perfringens* and related organisms know that no single test will confirm the presence of these organisms in a sample. For example, both *C. absonum* and *C. sardiniensis* produce stormy fermentation and can liquefy gelatin after several days. Similarly, heat-resistant strains of *C. perfringens* may not liquefy gelatin (8) and strains can lose the ability to liquefy gelatin. Thus, it is necessary to use stormy fermentation and gelatin liquefaction in RPM only as presumptive tests. All positive samples should be confirmed. *Clostridium perfringens* is identified as a nonmotile, gram-posi-

Table 1. Comparison of lactose gelatin medium and rapid perfringens medium for detection of gelatin hydrolysis by clostridia

Organism	Strain No.	Stormy fermentation in RPM	Gelatin hydrolysis	
			Lactose gelatin	RPM
Clostridium perfringens	FD-1	+	+	+
	FD-2	+	+	+
	FD-6	+	+	+
	FD-7	+	+	+
	FD-12	+	+	+
	FD-15	+	+	+
	FD-20	+	+	+
	FD-20	+	+	+
	FD-21	+	+	+
	FD-31	+	+	+
	FD-32	+	+	+
	FD-40	+	+	+
	FD-45	+	+	+
	A-86	+	−	−
	IU-3344	+	−	−
	IU-2825	+	−	−
	NCTC 10240	+	+	+
Clostridium paraperfringens	ATCC 27638	+	−	−
	ATCC 27639	+	−	−
Clostridium barati[a]	VPI 4446-2	+	−	−
Clostridium absonum	ATCC 27636	+	+	+
Clostridium sardiniensis	FD-0356	+	+	+
	FD-36	+	+	+

[a] Results in confirmatory media indicate that this strain is an atypical *C. perfringens*.

tive bacillus which produces black colonies in TSC agar, reduces nitrates to nitrites, produces acid and gas from lactose, produces acid from raffinose (9), and usually liquefies gelatin.

Acknowledgments

The authors thank James D. Macmillan, Rutgers University, New Brunswick, NJ, and Science Advisor, Food and Drug Administration, New York Regional Laboratory; and Stanley Harmon, Division of Microbiology, FDA, for invaluable assistance in the preparation of this paper.

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Mojonnier Method as Reference for Infrared Determination of Fat in Meat Products

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The Mojonnier method was compared with the conventional Soxhlet method for the determination of fat in 7 different meat products to assess its use as a standard reference method for calibration of commercial quantitative infrared transmission analyzers (e.g., Multispec M, Milkoscan 300 or 104). Results for the meat samples obtained by the Mojonnier method did not differ significantly from those obtained by the Soxhlet method. In addition, the Mojonnier method was less time-consuming and more precise than the Soxhlet; therefore, it can be used as a standard reference procedure for the calibration and assessment of infrared milk analyzers in their potential application to the rapid determination of fat in meat and meat products.

The determination of the basic chemical constituents such as fat, protein, and carbohydrate in food products has always been of importance to food processors for quality control. Use of traditional chemical methods for the determination of these 3 components is generally too slow and expensive for on-line compositional assessment. Infrared transmission spectroscopy has been successfully developed for quantitative determination of fat, protein, and lactose in milk and dairy products (1). Since its inception and acceptance as a rapid and quantitative method for milk analysis, advances in infrared instrumentation technology have led to the development of low cost and more reliable filter instruments (2, 3) affordable at the plant level for quality control. This success has led to the investigation of its application to other food products such as meat. The meat industry is one area where infrared analysis has great potential and work is presently ongoing in this laboratory to assess its accuracy and feasibility for fat and protein analysis.

The infrared method is based on establishing a relationship between the instrument signals obtained from component analysis (e.g., fat and protein) relative to the results obtained from a primary chemical reference method. By varying the levels of the individual components, a multicomponent relationship can be derived by multiple linear regression which accounts for cross-interference and water displacement effects. Once the instrument has been calibrated for a product in this manner, the uncorrected instrumental signals obtained from the samples to be analyzed are corrected with the derived equation to determine the component percentage.

The standard chemical reference method traditionally used for fat determination in meat is the Soxhlet method in which a meat sample is dried before solvent extraction. The use of infrared transmission instruments requires a different approach in sample preparation, i.e., the sample must be converted to a milk-like emulsion and the fat must be properly homogenized so that the particle size does not cause scattering of infrared radiation. Since calibration and accuracy of the instrument depend on the values assayed by the chemical reference method, the mode of fat determination, i.e., performed on an emulsion in one case and on a solid sample in the other, could significantly contribute to differences between results. The Mojonnier method is recognized as an accurate and precise standard method for determining fat content in milk and has been used for many years to calibrate infrared milk analyzers (4). To our knowledge, the Mojonnier method has not been routinely used for determining fat in meat and meat products. The objective of this research was to compare the Mojonnier and Soxhlet methods for determination of fat in meat samples and to assess the use of the former as a standard reference method for infrared analysis of meat and meat products.

Experimental

Seven commercially prepared meat products including ground pork, bologna, ham, turkey roll, and lean, medium, and regular ground beef were obtained at a local retail outlet. This selection provided a range of fat content and included both raw and processed meats. Each meat product was ground in a meat grinder 3 times using a 3.2 mm plate, and mixed thoroughly. Thirty gram balls were formed, frozen in liquid nitrogen, and stored at −20°C for use as required.

For the fat determination by Soxhlet, the

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Table 1. Mean [a] fat contents (%) obtained by Soxhlet and Mojonnier methods

Sample	Soxhlet	Mojonnier
Lean beef	12.34	12.27
Medium beef	20.19	20.30
Regular beef	26.48	26.64
Pork	25.30	25.43
Turkey roll	15.13	15.05
Ham	4.16	4.22
Bologna	27.55	27.26
Mean	18.74	18.74

[a] Six replicates. Standard error: Soxhlet, 0.28%; Mojonnier, 0.13%

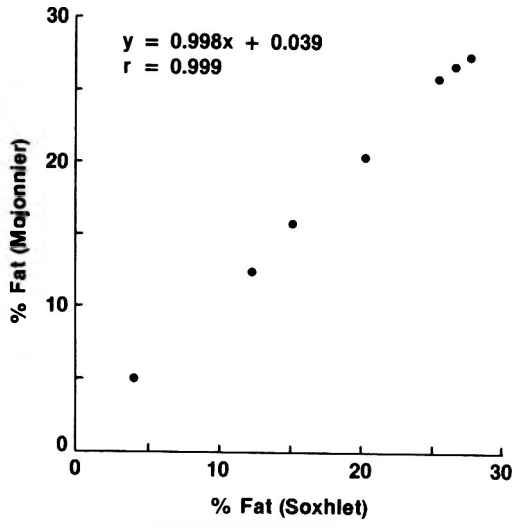

Figure 1. Plot of % fat by Mojonnier method vs Soxhlet method.

thawed meats were prepared and extracted according to AOAC method **24.005(b)** (5). The extraction was carried out for 4 h, using a condensation rate of about 5–6 drops/s, and the residual fat was calculated on a fresh weight basis.

The Mojonnier fat extraction was carried out with the Mojonnier extraction apparatus, using a procedure developed for milk (6). An emulsion was prepared by accurately weighing a 20 ± 0.01 g sample of thawed meat into a beaker, adding 100 g 0.1N NaOH, and heating in a water bath at 70°C for 5 min. The sample was then homogenized immediately using a Polytron mixer (Brinkmann Instruments, Rexdale, Ontario) at high speed for 4 min. The Polytron stem was washed with ca 50 mL prewarmed distilled water and the weight of the solution was brought to 200 g with more water. The meat emulsion prepared in this way served as a sample for the analysis of fat by the infrared analyzer and by the Mojonnier method. Ten grams of the homogeneous solution (representing 1 g meat) was taken for the Mojonnier extraction procedure. For the first extraction, 1.5 mL ammonia, 10 mL ethanol, 25 mL ethyl ether, and 25 mL petroleum ether were added to the sample, with shaking after each addition. For the second extraction, 5 mL ethanol and 25 mL each of ethyl ether and petroleum ether were added. For bologna, a precipitate formed with the above procedure was corrected by decreasing the ethanol to 6 and 3 mL in the first and second extractions, respectively, along with reversing the order of addition of ethanol and ethyl ether.

Results and Discussion

The mean fat content for all the meat samples by both the Soxhlet and Mojonnier methods is presented in Table 1. Analysis of variance of the individual methods indicated that there was no significant difference ($P < 0.05$) among the 6 replicates for each meat sample in either method. Better precision was obtained for the Mojonnier method which had a standard error (SE) of 0.13%; the SE for the Soxhlet method was 0.28%. The use of 20 g meat for the preparation of the emulsion provided a more representative sample compared with the 3–5 g sample used in the Soxhlet method, which could partly explain the lower SE for the Mojonnier results. Analysis of variance for the 2 methods indicated that the results obtained by Mojonnier did not differ significantly from the results obtained by Soxhlet. The relationship between the 2 methods can be seen from a plot of mean fat values in Figure 1. Linear regression analysis on all data yielded a slope of 0.998 and a correlation coefficient of 0.999. This one-to-one relation would indicate that the Mojonnier method could effectively substitute for the Soxhlet in determining fat content of the samples investigated in this study.

The Mojonnier method is recognized as an extremely reproducible method for milk (SE 0.02) which contains a relatively low fat content of 3.5%. Considering the wide range of fat content (4–26%), the diversity of meat samples, and the sampling difficulties associated with meat, a standard error of 0.13% is acceptable for Mojonnier results in this case. The Mojonnier method was simple and less time-consuming, requiring a maximum of 2 h for 8 samples, compared with

4 or more hours for the standard Soxhlet method.

Based on this study, it is suggested that for the purpose of calibrating or assessing the accuracy of infrared instrumentation relative to the fat content of meat products, the Mojonnier method be used as the reference method. The Mojonnier procedure has the distinct advantage of being performed on the actual sample presented to the instrument and hence allows a direct rather than indirect comparison of results. It may also provide a useful alternative to the standard determination of the fat content of meats in general, because of the considerable saving in time.

Acknowledgments

The authors thank the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Food and Agriculture for their financial support.

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Gas-Liquid Chromatographic Determination of Sucrose Fatty Acid Esters

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A method was developed for gas-liquid chromatographic determination of sucrose fatty acid esters as TMS derivative of sucrose and methyl esters of fatty acids. Sucrose fatty acid esters were completely degraded to sucrose and fatty acids in alkaline ethanol overnight at 25°C. Sucrose was derivatized with pyridine, trimethylchlorosilane, and *N*-trimethylsilylimidazole and the sucrose TMS derivative was determined on a 2% OV-17 column. Fatty acids were extracted with ethyl ether, methylated with BF_3-methanol complex at 65°C, and determined on a 2% DEGS + 0.5% H_3PO_4 column. This method was applied to selected sucrose fatty acid esters. For example, sucrose and fatty acids derived from 50 mg sample F20 were 10.6-11.0 and 38.1-39.0 mg, respectively. Total amounts were 48.7-50.0 mg with a standard deviation of 0.4 (n = 6).

In Japan, sucrose fatty acid esters are registered as a food additive and have been widely used not only in foods but also in drugs and detergents. Sucrose fatty acid esters are commonly determined by a colorimetric method based on the formation of hydroxamic acids (1). In the *Japanese Standards of Food Additives* (2), fatty acids are weighed after hydrolysis of the esters. But these methods cannot determine kinds and quantities of fatty acids of which the esters are composed.

Recently, sucrose monopalmitate has been determined by gas chromatography after trimethylsilylation (3), but it is difficult to determine sucrose di-, tri-, or polyesters as TMS derivatives by this method.

The present report presents a method that is applicable to all sucrose fatty acid esters and in addition can analyze fatty acids of which the esters are composed. Sucrose fatty acid esters are completely degraded to sucrose and fatty acids in alkaline ethanol solution and each is determined by gas chromatography after derivatization.

Received March 1, 1982. Accepted December 17, 1982.

METHOD

Apparatus

(a) *Hot dry bath.*—SHD-III (Iuchi Seieido Co., Ltd, Osaka, Japan).

(b) *Gas chromatograph.*—Yanaco G-80 (Yanagimoto Mfg Co., Ltd, Kyoto, Japan) with flame ionization detector.

Operating conditions: (*1*) For sucrose determination: temperatures (°C): column 230, injection port 280, detector 250; nitrogen flow ca 35 mL/min; 225 cm × 3 mm glass column packed with 2% silicone OV-17 on 80–100 mesh Chromosorb W (AW-DMCS). (*2*) For fatty acids determination: temperatures (°C): column 165, injection port 225, detector 225; nitrogen flow ca 40 mL/min; 225 cm × 3 mm glass column packed with 2% DEGS + 0.5% H_3PO_4 on 80–100 mesh Chromosorb W (AW-DMCS).

Reagents

(a) *Sucrose fatty acid esters.*—F20, F50, F90, F110, F160 (obtained from Daiichi Kogyo Seiyaku Co., Ltd, Kyoto, Japan). Samples were mixtures of different amounts of mono-, di-, tri-, and polysubstituted esters of stearic and palmitic acids.

(b) *Sucrose standard solutions.*—Stock solution: 500 μg/mL. Dissolve 50.0 mg sucrose analytical grade (Katayama Chemical Co., Ltd, Osaka, Japan) in 100 mL pyridine. Working solutions: Dilute aliquots of stock solution with pyridine to prepare solutions containing 25–250 μg sucrose/mL.

(c) *Fatty acids standard solutions.*—Stock solution: 1000 μg/mL. Dissolve 100 mg each of palmitic acid analytical grade (Nakarai Chemicals Ltd, Kyoto, Japan) and stearic acid analytical grade (Katayama Chemical Co., Ltd) in 100 mL ethyl ether. Working solutions: Dilute aliquots of stock solution with ethyl ether to prepare solutions containing 100–500 μg fatty acids/mL.

(d) *Internal standard solution for sucrose.*—Stock solution: 1000 μg/mL. Dissolve 100 mg *n*-octacosane analytical grade (Nakarai Chemicals Ltd) in 100 mL pyridine. Working solution: 100 μg/mL. Dilute 10 mL stock solution to 100 mL with pyridine.

(e) *Internal standard solution for fatty acids.*—Stock solution: 1000 μg/mL. Dissolve 100 mg *n*-eicosane standard kit (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan). Working solution: Dilute 25 mL stock solution to 100 mL with ethyl ether.

(f) *TMS reagent.*—Trimethylchlorosilane (TMCS), *N*-trimethylsilylimidazole (TSIM) (Nakarai Chemicals Ltd).

(g) *Methylation reagent.*—BF_3-methanol complex (Wako Pure Chemical Industries Ltd, Osaka, Japan).

Sample Preparation

(a) *Degradation of sucrose fatty acid ester.*—Weigh 50 mg ester into 100 mL volumetric flask, add 50 mL 4% NaOH ethanol solution, and let stand overnight at 25°C. Neutralize resulting solution with 1N HCl (BTB indicator), and dilute to volume with ethanol.

(b) *Derivatization of sucrose.*—Pipet 1 mL neutralized solution into 5 mL test tube with stopper, and evaporate to dryness 1 h under stream of nitrogen in 90°C dry bath. Add 1 mL pyridine, 0.2 mL TMCS, and 0.2 mL TSIM. Shake vigorously, and let stand 30 min at room temperature. Pipet 1 mL internal standard solution *n*-octacosane (100 μg/mL), and dilute to 3 mL with pyridine. Inject this sample solution into gas chromatograph.

(c) *Derivatization of fatty acids.*—Pour remaining neutralized solution into separatory funnel, add 100 mL water, acidify with 5 mL d-HCl, and extract twice with 50 mL ethyl ether. Combine ether layers in 100 mL volumetric flask, and dilute to volume with ethyl ether. Transfer an aliquot of this solution to 10 mL test tube with stopper, evaporate to dryness under stream of nitrogen at 40°C, and methylate 5 min at 65°C. Transfer this reaction mixture to 50 mL separatory funnel, add 15 mL saturated NaCl solution, and extract with 10 mL ethyl ether. Pipet 1 mL internal standard solution *n*-eicosane (250 μg/mL) into extract, and dilute to 10 mL. Inject this sample solution into gas chromatograph.

Determination of Sucrose

(a) *GLC determination.*—Inject 5 μL derivative solution into gas chromatograph, and plot relative peak height vs μg sucrose. Detection limit is ca 1.0 mg for sucrose.

(b) *Preparation of standard curve.*—Pipet 1 mL of each sucrose standard solution (25–250 μg/mL) into separate 5 mL test tubes with stoppers, and derivatize in same manner as sample.

Determination of Fatty Acids

(a) *GLC determination.*—Inject 5 μL derivative solution into gas chromatograph and plot relative peak height vs μg fatty acids. Detection limits are ca 0.5 mg for palmitic and stearic acids.

(b) *Preparation of standard curve.*—Pipet 1 mL of each fatty acids standard solution (100–1000 μg/mL) into separate 10 mL test tubes with stoppers and derivatize in same manner as sample.

Table 1. Components of selected sucrose fatty acid esters[a]

Sample (50 mg)	Sucrose, mg	Palmitic acid, mg	Stearic acid, mg	Total, mg
F 20	10.8 ± 0.2	11.0 ± 0.2	27.5 ± 0.2	49.3 ± 0.4
F 50	19.0 ± 0.2	5.8 ± 0.2	20.6 ± 0.6	45.4 ± 0.5
F 90	19.6 ± 0.4	8.0 ± 0.1	18.5 ± 0.3	46.1 ± 0.6
F 110	21.3 ± 0.4	7.3 ± 0.1	17.4 ± 0.3	46.0 ± 0.4
F 160	21.8 ± 0.5	8.3 ± 0.2	17.0 ± 0.2	47.1 ± 0.7

[a] Each value is the average of 6 trials ± SD.

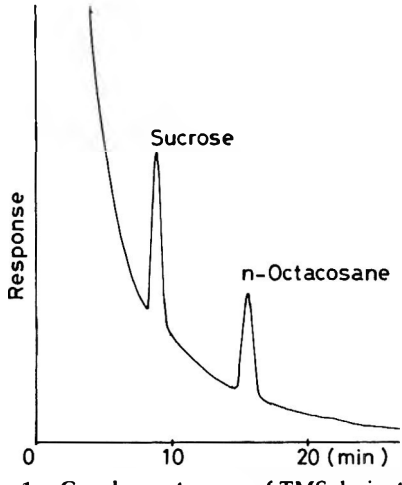

Figure 1. Gas chromatogram of TMS derivative of sucrose derived from sucrose fatty acid ester.

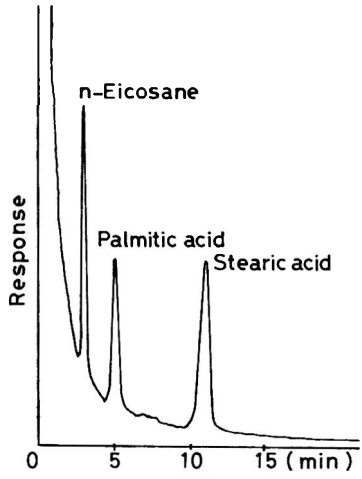

Figure 2. Gas chromatogram of methyl esters of fatty acids derived from sucrose fatty acid ester.

Results and Discussion

The degradation rate with 0.4–4% NaOH ethanol solutions was investigated. The optimum NaOH concentration was 4%. The degradation rate at 15, 25, 40°C was investigated; the optimum temperature was 25°C. The degradation rate was investigated over 20 h, and was constant over 16 h. Consequently, the degradation conditions were fixed as in *Sample Preparation*.

Effect of pH on decomposition of sucrose was investigated under the same conditions as evaporation of the neutralized solution to dryness (90°C, 1 h). Sucrose decomposition was nil at pH 6 ~ 9, so BTB indicator was used for neutralization.

A model solution was prepared by adding 50 mL ethanol, 3.0 g NaCl, and 10.0 mg sucrose to 150 mL water. To this solution, 10.0 mg each of stearic and palmitic acids were added, and extracted with ethyl ether in the same manner as in *Sample Preparation*. Average recoveries of the added palmitic and stearic acids were 101 and 97%, respectively (n = 3).

Gas chromatograms of TMS derivative of sucrose and methyl esters of fatty acids derived from Sample F20 are shown in Figures 1 and 2, respectively. Sample components were identified by comparison of GLC retention times. Standard curves for sucrose (25–250 μg) and fatty acids (100–1000 μg) gave straight lines.

Table 1 demonstrates the results of analyses of the esters. The complete degradation and accurate determination show the present method to be satisfactory for sucrose fatty acid esters analyses.

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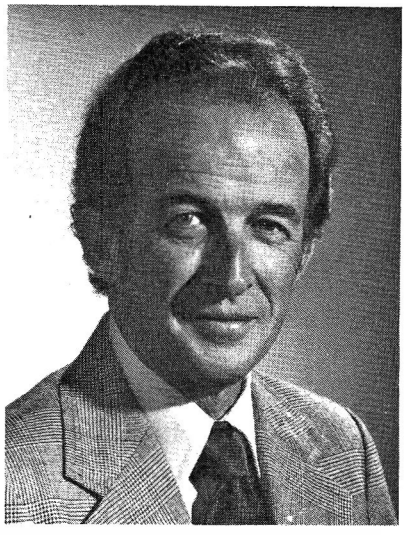

Wiley Award Winner, Velmer A. Fassel

Velmer A. Fassel, Deputy Director of the Ames Laboratory and the Energy and Mineral Resources Research Institute at Iowa State University, is the winner of the 1983 Harvey W. Wiley Award. Warren Bontoyan, 1983 AOAC President, will present the award to Dr. Fassel on Monday evening, October 4, at the AOAC 97th Annual International meeting.

Since 1956, the annual award has gone to a scientist who made an outstanding contribution toward developing and validating methods of analysis for foods, drugs, cosmetics, pesticides, feeds, fertilizers, environmental contaminants, or a related area. The $2500 award focuses attention on the role of the scientist in protecting the consumer and preserving the quality of the environment. It was established in honor of Harvey W. Wiley, "Father" of the 1906 Pure Food and Drug Act and a founder of AOAC.

In addition to directing the Ames Laboratory since 1969, Dr. Fassel is professor of chemistry at Iowa State University. He has presented more than 200 invited lectures at other universities, research institutes, and national and international conferences. In 1976, he was named Distinguished Professor of Science and Humanities.

Throughout his career, Dr. Fassel has made major contributions toward the field of analytical chemistry. He developed the methods to analyze rare earth mixtures, and his flame spectrometric methods brought about vast improvements in the use of flame emission spectrometry. He has published more than 150 research reports on atomic emission, absorption, and fluorescence spectroscopy. The award honors his work on induction-coupled plasma spectrometry and on the theory of atomic spectral analysis.

Dr. Fassel's achievements have been recognized by various professional organizations. He was elected to Fellow membership by the American Association for the Advancement of Science and the Optical Society of America. He became an honorary member of the Japan Society for Analytical Chemistry in 1981 and of the Society for Applied Spectroscopy in 1982. In 1967, he was appointed to the Joint Commission on Spectroscopy of the International Council of Scientific Unions. Dr. Fassel has also served as both chairman and secretary of the International Union of Pure and Applied Chemistry's Commission on Spectrochemical Methods of Analysis. He is a member of the American Chemical Society, the American Institute of Physics, and the Iowa Academy of Sciences.

Dr. Fassel has received many awards, including the Annual Medal Award of the Society for Applied Spectroscopy (1964), the Spectroscopy Society of Pittsburgh Award (1969), the Maurice F. Hasler Award sponsored by Bausch and Lomb (1971), and the Anachem Award of the Association of Analytical Chemists (1971). He served as a co-editor of the journal *Spectrochimica Acta* for 13 years, and was given a special gold medal in appreciation for his work.

He received his Ph.D. degree from Iowa State University in 1947, after which he became both assistant professor in the university's chemistry department and associate chemist in the Ames Laboratory. He has a B.A. degree from Southeast Missouri State University.

Meetings

October 2–6, 1983: 97th Annual International Meeting of the AOAC, Shoreham Hotel, Washington, DC. Contact Kathleen Fominaya, AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209; telephone 703/522-3032.

November 6–9, 1983: 4th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Hyatt Regency Hotel, Crystal City, Arlington, Virginia: "Multidisciplinary Approaches to Environmental Problems."

Contact SETAC, PO Box 352, Rockville, MD 20850; telephone 301/468-6704.

February 13–17, 1984: International Conference on Oils, Fats and Waxes, University of Auckland, Auckland, New Zealand. Contact S. G. Brooker, Chemistry Department, University of Auckland, Private Bag, Auckland, New Zealand.

March 28–30, 1984: Challenges to Contemporary Dairy Analytical Techniques, University of Reading, Reading, UK, sponsored by International Dairy Federation, Federation of European Chemical Societies Working Party on Food Chemistry, and Association of Official Analytical Chemists. Contact Seminar Secretariat E. Wellingham, Royal Society of Chemistry, Burlington House, London, W1V OBN, UK.

April 30–May 2, 1984: AOAC 9th Annual Spring Training Workshop, Philadelphia Marriott Hotel, City Line Avenue, Philadelphia, PA. Kickoff for AOAC's Centennial Celebration. Contact James Karr, Pennwalt Corp., 900 First Ave, King of Prussia, PA 19406, phone 215/265-3200; or Harvey Miller, Food and Drug Administration, 2nd and Chestnut Sts, Philadelphia, PA 19106, phone 215/597-4375.

AOAC Supporters

The following companies have been added to the list of Private Sustaining Members of AOAC: Dow Chemical Company, Midland, MI; Timothy S. Stevens, Representative. 3M Medical Products Division, St. Paul, MN; Gary Krejcarek, Representative. Life Savers, Inc., Port Chester, NY; Harry Jacin, Representative. Tastybird Foods, Russellville, AR; Robert E. Waggoner, Representative.

New Environmental Standard Reference Material (SRM)

Priority Pollutant Polynuclear Aromatic Hydrocarbons (in Acetonitrile) SRM 1647 is intended for calibrating chromatographic instrumentation used in the determination of the polynuclear aromatic hydrocarbons (PAHs) certified in this SRM. It is also useful in recovery studies for adding known accurate amounts of these PAHs to a sample; because of its miscibility with water, it can be used to fortify aqueous samples with known concentrations of PAHs. Unit: 6 mL; $125. Available from Office of Standard Reference Materials, Room B311, Chemistry Bldg, National Bureau of Standards, Washington, DC 20234.

Pattern Recognition Programs

Pattern recognition programs, SIMCA 3B, written for 8080 or Z-80 (trademark Zilog, Inc.) microcomputers using the CPM (Digital Research) operating systems are available on 8 in. single density floppy disks. The programs developed by S. Wold and M. Sjostrom (ACS Symposium Series No. 52, 1977) are powerful tools for the analysis of chemical data in terms of generalized pattern recognition. This approach has much to offer analytical chemists in developing quality control programs associated with complex mixtures, assessment of information obtained from large numbers of samples involving many peaks, and evaluating relationships among variables in complex mixtures. The program and manuals are available for $750 from Principal Data Components, 2505 Shepard Blvd, Columbia, MO 65201, phone (314) 449-0312. Copies of documentation are also available for $35 per copy, with cost credited to purchase price of software.

Interim Methods Adopted

The following methods have been adopted interim official first action by approval of the appropriate General Referee, Committee on Official Methods, and Chairman of the Official Methods Board: Total Vitamin C in Food, Semiautomated Fluorometric Method, by J. W. DeVries, General Mills, Inc., Dalapon Grasskiller Products, Liquid Chromatographic Method, by T. S. Stevens and C. Wedelstaedt, Dow Chemical U.S.A. Titratable Acidity in Corn Syrup, by R. Bernetti and R. Owen, Corn Refiners Association. Gas Chromatographic Determination of Ethanol in Beer, ASBC method submitted by A. J. Cutaia, The Stroh Brewery Co.; Gas Chromatographic-TEA Determination of *N*-Nitrosodimethylamine in Nonfat Dry Milk, by N. P. Sen, S. Seaman, and R. Stapley, Health and Welfare Canada; Gas Chromatographic Determination of Fentin in Fentin-Maneb Preparations, CIPAC method previously published by J. Basters et al. (*J. Assoc. Off. Anal. Chem.* **61,** 1507 (1978). These methods will be considered for adoption official first action at the 97th Annual International Meeting, October 1983. Copies of the methods are available from the AOAC, 1111 N 19th St, Suite 210, Arlington, VA 22209.

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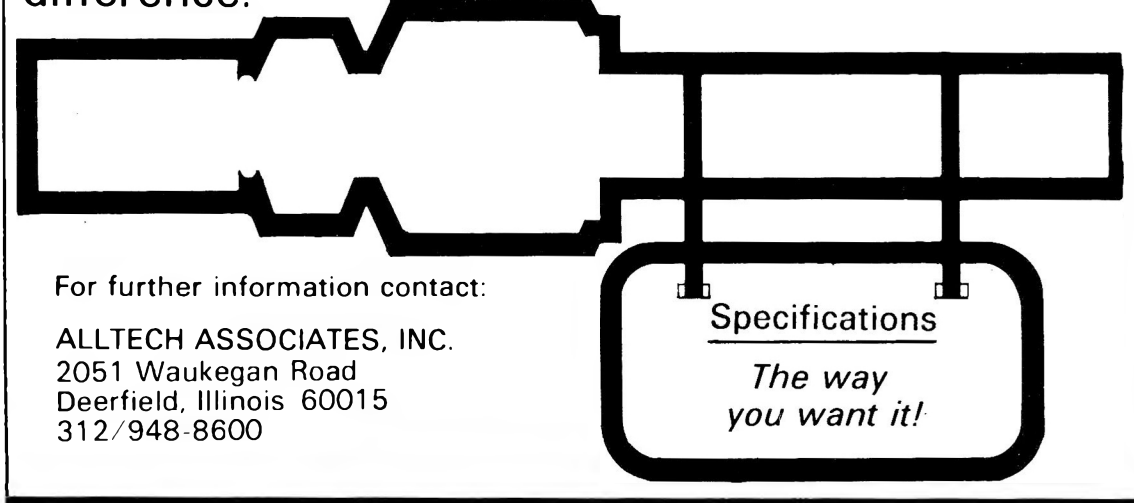

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