ISSN 0021-9673

VOL. 503 NO. 2 MARCH 23, 1990

THIS ISSUE COMPLETES VOL. 503

# JOURNAL OF CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS



ELSEVIER

## JOURNAL OF CHROMATOGRAPHY

**Scope.** The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis or alterations during medical treatment; screening and profiling of body fluids or tissues with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; drug level monitoring and pharmacokinetic studies; clinical toxicology; analytical studies in occupational medicine.

**Submission of Papers.** Papers in English, French and German may be submitted, in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Review articles are invited or proposed by letter to the Editors. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

**Subscription Orders.** Subscription orders should be sent to: Elsevier Science Publishers B.V., P.O. Box 211, 1000 AE Amsterdam, The Netherlands, Tel. 5803 911, Telex 18582 ESPA NL. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

**Publication.** The *Journal of Chromatography* (incl. *Biomedical Applications*) has 37 volumes in 1990. The subscription prices for 1990 are:

*J. Chromatogr.* (incl. *Cum. Indexes, Vols. 451–500*) + *Biomed. Appl.* (Vols. 498–534):
Dfl. 6734.00 plus Dfl. 1036.00 (p.p.h.) (total *ca.* US$ 3885.00)

*J. Chromatogr.* (incl. *Cum. Indexes, Vols. 451–500*) only (Vols. 498–524):
Dfl. 5616.00 plus Dfl. 756.00 (p.p.h.) (total *ca.* US$ 3186.00)

*Biomed. Appl.* only (Vols. 525–534):
Dfl. 2080.00 plus Dfl. 280.00 (p.p.h.) (total *ca.* US$ 1180.00).

Our p.p.h. (postage, package and handling) charge includes surface delivery of all issues, except to subscribers in Argentina, Australia, Brasil, Canada, China, Hong Kong, India, Israel, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand and the U.S.A. who receive all issues by air delivery (S.A.L. — Surface Air Lifted) at no extra cost. For Japan, air delivery requires 50% additional charge; for all other countries airmail and S.A.L. charges are available upon request. Back volumes of the *Journal of Chromatography* (Vols. 1–497) are available at Dfl. 195.00 (plus postage). Claims for missing issues will be honoured, free of charge, within three months after publication of the issue. Customers in the U.S.A. and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 655 Avenue of the Americas, New York, NY 10010. Tel. (212) 633-3750.

**Abstracts/Contents Lists** published in Analytical Abstracts, ASCA, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Clinical Chemistry Lookout, Current Contents/Life Sciences, Current Contents/Physical, Chemical & Earth Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Pharmaceutical Abstracts, Referativnyi Zhurnal, Science Citation Index and Trends in Biotechnology.

**See inside back cover** for Publication Schedule, Information for Authors and information on Advertisements.


---




0021-9673/90/$03.50





Printed in The Netherlands

# CONTENTS

*(Abstracts/Contents Lists published in Analytical Abstracts, ASCA, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Chromatography Abstracts, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Excerpta Medica, Index Medicus, Mass Spectrometry Bulletin, PASCAL-CNRS, Referativnyi Zhurnal and Science Citation Index)*

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

# Determination of Beta-Blockers in Biological Material

*edited by* **V. Marko**, *Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Bratislava, Czechoslovakia*

**(Techniques and Instrumentation in Analytical Chemistry, 4C)**

This is the third volume of a sub-series entitled *Evaluation of Analytical Methods in Biological Systems*. (The first two were *Analysis of Biogenic Amines* edited by G.B. Baker and R.T. Coutts and *Hazardous Metals in Human Toxicology* edited by A. Vercruysse). This new volume addresses beta-blockers - an area of research for which a Nobel Prize in Medicine was awarded in 1988. It provides an up-to-date and comprehensive coverage of the theory and practice of the determination of beta-blockers in biological material. Two main fields of research are dealt with in this book: analytical chemistry and pharmacology, and, as it deals with drugs used in clinical practice, it is also related to a third area: therapy. Thus, it offers relevant information to workers in all three fields.

Some 50 beta-blockers and nine methods of analysis are discussed. The methods are divided into three groups: optical, chromatographic, and saturation methods. In addition to the analytical methods themselves, sample handling problems are also covered in detail, as is the information content of the analytical results obtained. Special chapters are directed to those working in pharmacology and pharmacokinetics. Finally, as recent evidence points to the increased importance of distinguishing optical isomers of drugs, a chapter on the determination of optical isomers of beta-blockers in biological material is also included. An extensive subject index and two supplements giving retention indices and structures of beta-blockers complete the book.

This is the first book to treat beta-blockers from the point of view of their determination and to discuss in detail the use of analytical methods for beta-blockers. It will thus appeal to a wide-ranging readership.



**1989 xiv + 334 pages**
**US$ 152.75 / Dfl. 290.00**
**ISBN 0-444-87305-8**

**ELSEVIER SCIENCE PUBLISHERS**

**P.O. Box 211, 1000 AE Amsterdam, The Netherlands**
**P.O. Box 882, Madison Square Station, New York, NY 10159, USA**

# NATURAL PRODUCTS ISOLATION

## Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors

*edited by* **GERALD H. WAGMAN** *and* **RAYMOND COOPER,**
*Schering-Plough Research, Bloomfield, NJ, USA*

## (Journal of Chromatography Library, 43)

This new book encompasses, in great detail, the most recent progress made in the isolation and separation of natural products. Written by experts in their respective fields, it covers antibiotics, marine and plant-derived substances, enzyme inhibitors and interferons. The book has extensive isolation schemes, tables, figures and chemical structures. In many instances a short summary of the producing organism, brief chemical description and structure and biological activity of the compounds is presented. Detailed information of extraction, separation and purification techniques follow. Each chapter has an extensive bibliography and, where applicable, an appendix showing sources of materials and equipment. A detailed index to the subject matter is also provided.

The book thus offers the reader: up-to-date reviews (including 1988) of specific topics in the natural products field not to be found elsewhere; information on new chromatographic methods and techniques described in sufficient detail to be utilized by investigators in this area of research; and extensive references to enable the serious researcher to pursue particular information. It will appeal to pharmaceutical and natural products researchers and is a valuable acquisition for university chemistry and biochemistry departments.

**Contents:** Countercurrent chromatography *(J.B. McAlpine, J.E. Hochlowski)*. HPLC detection methods for microbial products from fermentation broth *(R. Mierzwa et al.)*. Affinity and purification of glycopeptide antibiotics *(R.D. Sitrin, G.F. Wasserman)*. Nikkomycins and polyoxins *(H.-P. Fiedler)*. Saframycins and isoquinolines *(T. Arai)*. New cephalosporins *(S. Harada)*. Monocyclic β-lactam antibiotics *(W.L. Parker)*. Isolation of carbapenems *(K.E. Wilson)*. Avermectins and related compounds *(T. Miller, V.P. Gullo)*. Bioactive compounds from marine organisms and cultivated blue-green algae *(J.S. Mynderse et al.)*. The interferons *(S. Pestka)*. Enzyme inhibitors produced by microorganisms *(H. Umezawa)*. Alkaloidal glycosidase inhibitors from plants *(L.E. Fellows, G.W.J. Fleet)*. Chemical communication and control of development *(C.E. Smith, J.D. Orr, D.G. Lynn)*. Subject Index.

*1989 xii + 618 pages*
*US$ 150.00 / Dfl. 285.00*
*ISBN 0-444- 87147-0*

**ELSEVIER SCIENCE PUBLISHERS**

**P.O. Box 211, 1000 AE Amsterdam, The Netherlands**
**P.O. Box 882, Madison Square Station, New York, NY 10159, USA**

*Journal of Chromatography*, 503 (1990) 293–357
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 145

# Review

# Chromatographic analysis of chemical warfare agents

Z. WITKIEWICZ*, M. MAZUREK and J. SZULC
*Institute of Chemistry, Military Technical Academy, 01 489 Warsaw 49 (Poland)*
(First received July 11th, 1989; revised manuscript received November 10th, 1989)

CONTENTS



## 1. INTRODUCTION

Chemical weapons continue to be used in armed conflicts[1], although various treaties have been or are being negotiated[2]. Hence adequate analytical methods are required that would allow verification that treaties on the prohibition of chemical weapons are observed[3–5]. In this respect, a research project has been established in Finland on the identification and determination of over 100 warfare agents and 86 products of their degradation[6–16], and a study of this and related problems has been

0021-9673/90/$03.50 

made by a group of Canadian researchers on the order of the Secretary General of UNO[17].

The problems connected with the determination of substances classified as potential warfare agents lie also in the non-military sphere of interest. This concerns, for instance, the uncontrolled spread of toxic substances as a result of industrial breakdown or agrotechnical operations, and the generation of poisons, *e.g.*, fluoroacetic acid in plants or phosgene in the troposphere[18–20].

The detection and determination of highly toxic substances in complex environmental and biological systems by conventional chemical and biochemical methods is difficult and time-consuming, and the results are often dubious. These methods are now being systematically replaced by instrumental analytical methods, among which chromatographic procedures play an important role. The latter are distinguished by their high detectability, rapidity and the possibility of operation in a continuous mode. Chromatographic methods allow the isolation of analytes from complex matrices and their identification and determination even at picogram levels.

The number of publications on the determination of chemical warfare agents by chromatographic methods is considerable, but none of the chromatographic systems is universal, as they do not allow the analysis of all compounds simultaneously and under the same conditions. This is to be expected, as the main property that allows the classification of a substance as a warfare agent is its toxicity and applicability on the battle field[21]. The various chemical warfare agents differ considerably in their physico-chemical properties, *e.g.*, polarity and boiling point, which are decesive for chromatographic separations. The problems connected with the selection of chromatographic systems become even more complicated as it is necessary also to take into account the degradation of warfare agents, the starting materials used for their synthesis and contaminants.

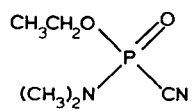
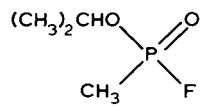
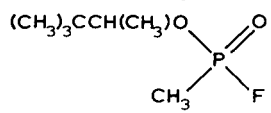
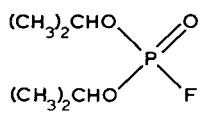
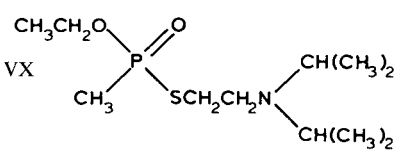
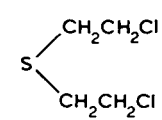
Hitherto several surveys have been published on the applications of chromatography in the analysis of chemical warfare agents, but their approach was superficial[4,22,23]. In this review, an attempt is made to survey comprehensively the possibilities of applying modern chromatographic methods in the analysis of chemical warfare agents. We therefore consider thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC), and survey their applications in the analysis of the following types of chemical warfare agents: organophosphorus [tabun (GA), sarin (GB), soman (GD), DFP and VX]; vesicants [mustard gas (HD), nitrogen mustard (HN-3) and lewisite (L)]; irritants [2-bromobenzylnitrile (CA), 2-chloroacetophenone (CN), dibenz[*b, f*]-1,4-oxazepine (CR), *o*-chlorobenzylidenemalononitrile (CS), adamsite (DM) and chloropicrin (PS)]; psychotoxic [3-quinuclidinylbenzylate (BZ)]; and industrial [cyanogen chloride (CK), hydrocyanic acid (AC), phosgene (CG), fluoroacetic acid and sodium fluoroacetate]. The formulae and physico-chemical properties of these substances are given in Table 1.

## 2. METHODS OF COLLECTING AND PREPARING SAMPLES OF CHEMICAL WARFARE AGENTS FOR ANALYSIS

A sample after collection should have a composition representative of that of the original contaminated material, *i.e.*, the quantitative proportions of the components in the collected sample and in the initial bulk material should be identical.

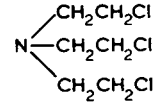
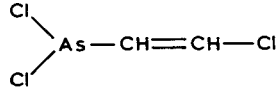
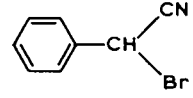
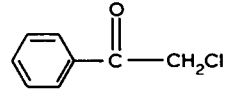
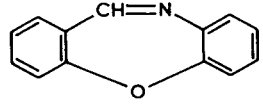
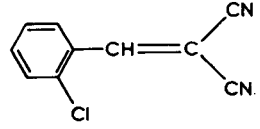
TABLE 1

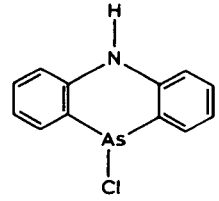
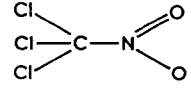
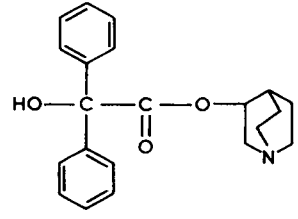
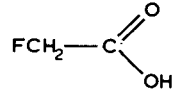
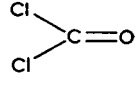
SELECTED PHYSICO-CHEMICAL PROPERTIES OF CHEMICAL WARFARE AGENTS

| *Chemical name* | *Common name* | *Abbreviation*[a] | *Structure* | *Molecular weight* | *m.p. (°C)* | *b.p. (°C)* | *Ref.* |
|---|---|---|---|---|---|---|---|
| Ethyl N,N′-dimethylphosphoroamidocyanidate | Tabun | GA |  | 162.1 | −50 | 230[b] | 24 |
| Isopropyl methylphosphonofluoridate | Sarin | GB |  | 140.1 | −54 | 151.5[b] | 24 |
| Pinacolyl methylphosphonofluoridate | Soman | GD |  | 182.1 | −80 | 167[b] | 24 |
| Diisopropyl phosphorofluoridate | DFP | PF-3 |  | 184.2 | −82 | 183[b] | 11 |
| O-Ethyl, S-2-diisopropylaminoethyl methylphosphonothiolate | VX | VX |  | 267.3 | −30 | >300[b] | 24 |
| Bis(2-chloroethyl)sulphide | Mustard gas yperite | HD |  | 159.1 | 14.5 | 217[b] | 24 |

*(Continued on p. 296)*

TABLE 1 (*continued*)

| *Chemical name* | *Common name* | *Abbreviation*[a] | *Structure* | *Molecular weight* | *m.p. (°C)* | *b.p. (°C)* | *Ref.* |
|---|---|---|---|---|---|---|---|
| Tris(2-chloroethyl)amine | Nitrogen gas | HN-3 |  | 204.5 | −4 | 235[b] | 24 |
| 2-Chlorovinyldichloroarsine | Lewisite[c] | L |  | 207.3 | −2.4 | 196.6[b] | 24 |
| 2-Bromobenzylnitrile | Camite | CA |  | 196.0 | 25 | 242[b] | 24 |
| 2-Chloroacetophenone | | CN |  | 154.5 | 59 | 245 | 24 |
| Dibenz[*b*,*f*]-1,4-oxazepine | | CR |  | 195.2 | 71–72 | 300 | 24 |
| 2-Chlorobenzylidenemalonodinitrile | | CS |  | 188.6 | 95–96 | 310–315 | 11 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10-Chloro-5,10-dihydrophenarsazine | Adamsite | DM |  | 277.5 | 195 | 410 | 24 |
| Trichloronitromethane | Chloropicrin | PS |  | 164.4 | −64 | 112.3 | 24 |
| 3-Quinuclidinylbenzylate | | BZ |  | 337.4 | 164–165 | 320 | 24 |
| Fluoroacetic acid | | |  | 78.0 | 33 | 167 | 25 |
| Hydrocyanic acid | | AC | HCN | 27.0 | −13.3 | 25.7 | 24 |
| Cyanogen chloride | | CK | ClCN | 61.5 | −6.9 | 12.6 | 24 |
| Carbonyl chloride | Phosgene | CG |  | 98.9 | −118 | 8.2 | 24 |

[a]Notation used in U.S. Army.
[b]Degradation of compound.
[c]Data for *trans* isomer.

The chromatographic separation proper is usually preceded by the isolation of the substances to be determined from a sample collected directly from the environment[26]. Application of sensitive selective detectors often allows some stages of preparation of the sample to be omitted, but even very small amounts of contaminants may sometimes occur that make an acceptable determination difficult or even impossible. It is often necessary to subject the toxic substances to be determined to repeated concentration[27].

A method that allows the isolation of the substance to be analysed from the condensed (liquid or solid) matrix is the headspace procedure[28–31], which consists in analysing the vapour phase of substances in thermodynamic equilibrium with the same substances in the condensed phase. There are two modifications of the method. In the first, vapour (*e.g.*, of chloropicrin[32]) is collected from over the condensate placed in a closed vessel after phase equilibrium has been established. The second modification consists in passing an inert gas over the condensed phase, vapour of the analyte being entrained and subsequently analysed chromatographically. The dynamic modification has a higher sensitivity and, when the system is kept under constant conditions, the concentration of the component to be determined varies almost linearly with the volume of the inert gas passed, which facilitates quantitative analysis. This procedure has been used to isolate certain chemical warfare agents that decomposed in the first modification (*e.g.*, soman[9]).

To accelerate the establishment of equilibrium in the system in the headspace method, the temperature is increased, the liquid phase is salted-out or the surface of the condensed phase is expanded. The headspace technique may be used to determine substances whose boiling points lie in a wide range, *e.g.*, from 26°C (HCN) to 178°C (methylheptanol)[33].

The vapour from over the condensed phase may be analysed directly after it has been introduced in an adequate volume into the chromatographic column. One can also arrange that the sample from the equilibrium vessel is introduced into the chromatographic column with a cooled section where the sample components undergo sorption on the stationary phase; their separation starts when cooling is discontinued.

Much attention has been devoted to sample preparation for chromatographic analysis and new devices and instrumentation have been designed for this purpose[34]. Nevertheless, a survey of methods for collecting samples of materials contaminated with chemical warfare agents shows that no universal method exists. The choice of a method depends on the kind of agent and contaminated material, and also on the purpose of the analysis. The problems of the reliability of the results of the analysis of toxic compounds related to the collection of samples have been discussed in the literature[4,6,9,10,35–37]. The methods of isolating chemical warfare agents from various media are given below in greater detail.

### *2.1. Air*

Sample collection of chemical warfare agents from air is carried out mainly by absorption and adsorption methods[38,39], which make possible the simultaneous concentration of the compounds to be analysed.

Absorption methods consist in passing contaminated air through a solvent, mixture of solvents or solution in which the toxic compound dissolves, sometimes with the formation of its derivatives[40–43]. By applying solvents with a high boiling

point with good absorption of the analyte substances, trace amounts of warfare agents present in air can be concentrated. To increase the efficiency of absorption of lower boiling components, cooling of the sorption system with ice, dry-ice or liquified gases is often applied[13,44]. The solution of the toxic substance obtained as a result of absorption is often suitable for direct use in chromatographic analysis.

If it is necessary for the measurements proper to be preceded by additional concentration of trace amounts of the toxic agent to a level corresponding to the sensitivity of the measuring system, then, depending on the physico-chemical properties of the substances being analysed, distillation or extraction is applied[45]. Distillation is usually used when the sample contains components that differ considerably in volatility, whereas extraction is applied when the components have similar volatilities but different solubilities.

Distillation permits the separation of volatile organic substances from the non-volatile residue. To reduce the effects connected with the chemical derivatization of the compounds to be analysed (due to heating), distillation is sometimes conducted in a stream of inert gas and under reduced pressure. The volatile components are collected in adsorption columns or condensed in receivers[46,47].

Extraction consists in washing the dissolved sample with small volumes of a solvent selected so that it be immiscible with the sample solvent and that the partition coefficients of the components being analysed be higher than those of the matrix components. If necessary, the extract obtained is concentrated by evaporation of the solvent, sometimes in an inert gas (*e.g.*, nitrogen) atmosphere[9,32,48].

Adsorption is the fundamental technique used in the collection of samples of the substances to be analysed from air[6,9,10,49]. This process is carried out in samplers filled with adsorbents such as active carbon, silica gel, Tenax GC, porous polymers (Porapak Q and N, Chromosorb 102), XAD resins (XAD-1, -2, -4, -7) or polyurethane foam. Most of these materials are used as fillings of chromatographic columns[50–56]. Note that it is not recommended to use active carbon for the adsorption of organophosphorus compounds as it may induce their decomposition[9]. To concentrate a mixture whose components differ significantly in volatility, complex systems are used, composed of layers of different adsorbents, *e.g.*, polyurethane foam and XAD-2[13], Ambesorb, Chromosorb[104] and Tenax GC[56], or samplers filled with a mixture of adsorbents[57–59].

Air humidity may hinder significantly the process of sorption on the adsorbent, so various drying agents (*e.g.*, magnesium perchlorate) are used. They are placed directly before the adsorption sampler[48,60].

The chemical warfare agents are transferred from the adsorbent to the liquid or gas phase by applying extraction in the liquid–solid system or thermal desorption[52].

Extraction is the most common method of transferring the chemical warfare agents from the adsorbent into solution. Its advantage is the possibility of obtaining concentrated components in liquid form suitable for direct analysis by any chromatographic procedure (GC, HPLC, TLC).

Thermal desorption of the components trapped on the adsorbent is used when analysis is carried out by GC. This method allows almost 100% recovery of the adsorbed, thermally stable compounds[61], and the detectable concentrations of chemical warfare agents are 2–3 orders of magnitude lower than when extraction is used[56]. Tenax GC, characterized by a high thermal stability (up to 375°C) and resistance to

hydrolysis, proved to be the best sorbent for application in the desorption technique[9,56]. When the boiling temperatures of the sample components differ substantially, it may be necessary to separate them by desorption and repeated adsorption of the more volatile components. The desorption of these components is conducted for 10–15 min in a stream of inert (carrier) gas, then they are adsorbed in an adsorption column preceding the gas chromatograph column or trapped in few initial cooled coils of the capillary chromatographic column. The subsequent rapid (several dozen seconds) heating of these intermediate traps makes it possible to introduce the sample into the main column without producing excessive diffusion of the peak fronts of the chromatographed substances[62–66].

In the analysis of chemical warfare agents, it is not recommended to use glass or metal vessels for contaminated air samples, as irreversible adsorption of these agents on the vessel walls or even their decomposition may occur. The use of inert materials such as PTFE or polyethylene is to be preferred[67].

Aerosols are collected on filter-paper or other filters with suitable pore diameters[68]. From the military point of view, the most important is the aerosol fraction with particles of diameter not exceeding 5 $\mu$m, as this fraction has the ability to remain suspended for long periods in the layers of air close to the earth's surface. An assembly for the two-step isolation of chemical warfare agents from air has been presented[10]. In this assembly the aerosols are arrested on a Whatman GF/A glass-fibre filter and the gases and vapours in an adsorption column filled with XAD-2 resin or active carbon. This assembly was used for collecting air samples from an aircraft[69].

In the West-German MM-1 field gas chromatograph combined with a mass spectrometer, the sample is concentrated with the help of selective silicone membranes[70]. The latter adsorb organic pollutants from air, allowing the simultaneous diffusion of chemical warfare agents into the chromatographic column. The diffusion is accelerated by heating the membrane. This procedure is also used in the analysis of water pollutants.

### *2.2. Water*

In water analysis, the dissolved chemical warfare agents are isolated mainly by extraction or adsorption[71,72] methods or by a combination of both[73]. Less frequently, although to an increasing extent, the headspace method is also used.

For extraction, commonly available solvents are usually used[74–78]. In order to increase the partition coefficients of the substances being extracted between the two liquid phases, neutral salts are often added[79,80], *e.g.*, for the extraction of organophosphorus compounds[77].

In the adsorption method, columns are used filled usually with XAD-2, -4 or -7 resin[81,82]. The structure of these resins allows the sorption of organic compounds in the micropores without offering a greater resistance to water flow. The adsorption of the toxic agent is the greater the higher is its molecular weight and the greater its hydrophobicity. The kind of the resin used depends on the polarity of the compound being isolated. It has been shown that XAD-2 resin can be used for the quantitative isolation from water of many classes of compounds at concentrations ranging from $10^{-5}$ to $10^{-6}$%. For pesticides, present in water at a concentration of the order of $10^{-10}$%, the recovery achieved was 80–95%[56]. For most chemical warfare agents it is suggested that XAD-4 resin is used[9,10]. Adsorptive materials such as Porapak, Tenax

GC, μBondapak $C_{18}$, polyurethane foams and graphitized carbon black are also used[56,83–85].

In order to enrich samples containing trace amounts of the analysed components, lyophilisation is sometimes applied. For this purpose a salt, *e.g.*, sodium chloride, is added to the water being analysed and the system is subjected to freezing. Next water is removed by sublimation of ice. The residue contains the salt and the chemical warfare agents.

In a different procedure, an organic solvent is added to the water and, after freezing out ice, the organic phase is removed. This method of enriching the sample is suitable for treating solutions of concentrations lower than 0.01 mol/l. At higher concentrations losses of the component being determined may occur due to its occlusion on the forming ice[36].

It is often advantageous to subject the sample components to chemical derivatization prior to their isolation. Owing to the presence in their molecules of polar groups and their high molecular weights, many organic compounds are of low volatility and on heating undergo thermal decomposition or intramolecular rearrangement. By derivatization such as acetylation, methylation, perfluoroacetylation or silylation one can increase the volatility of the compounds and, as a result, facilitate their chromatographic analysis. An exhaustive survey of methods for the derivatization of compounds prior to their chromatographic analysis was made by Blau and King[86].

An interesting concept of combining extraction and derivatization in one process was advanced by Rosenfeld *et al.*[87]. XAD-2 resin impregnated with benzyl or pentafluorobenzyl bromide was used. The impregnants caused derivatization of the organic acids adsorbed from water. It seems that this method could be used successfully in the analysis of decomposition products of organophosphorus compounds by gas or liquid chromatography.

### *2.3. Soil*

Most methods of collecting samples of soil are fairly complex. In principle, they are useful only with respect to chemically stable chemical warfare agents which are resistant to degradation reactions caused by the influence of the environment[88,89]. The most common method of isolating the compounds to be analysed from soil is their extraction with organic solvents, preceded by preliminary wetting of the soil with water[90,91].

De Leeuw *et al.*[92] described a method of isolating volatile and medium volatile substances from soil. It consists in direct evaporation of the substances to be analysed in a pyrolyser by means of a metal wire which is heated rapidly (0.1–0.2 s). The compounds liberated due to heating or generated in the course of pyrolysis are passed through a capillary column in which they are separated. Good reproducibility of results of analysis was achieved with this method.

Some chemical warfare agents present in soil may be analysed by the headspace technique.

### *2.4. Vegetable material*

In phytochemical analysis, the residues of toxic substances are usually isolated by simple methods. In most instances the sample is homogenized and the components to be analysed are extracted in a Soxhlet apparatus with a mixture of organic sol-

vents[90,93–100]. The extract obtained is usually dried, *e.g.*, with sodium sulphate, and filtered through a Whatman filter-paper.

If the samples contain wax in amounts greater than 15%, the latter is removed preliminarily by treating the sample with a non-polar solvent or, depending on the sample composition, the components to be analysed are isolated without removing the wax by using more polar solvents, *e.g.*, a mixture of acetonitrile, benzene and hexane[101].

To ensure a better separation of the contaminants from the compounds being determined, the extract obtained is passed through a column filled with active carbon, aluminium oxide or Celite[102,103]. The process of separation of the components of interest from the contaminants extracted with them is controlled by selection of a suitable adsorbent. For this purpose thin-layer chromatography may also be used[93,94].

### *2.5. Samples for determining the contamination of humans and animals*

The degree of contamination of humans and animals is usually assessed by analysing body fluids such as blood, plasma or urine. The concentration of a chemical warfare agent in blood and plasma is representative of the mean concentration of that agent in the whole organism. In contrast, samples of urine sometimes show significant differences in the concentration of the chemical warfare agents being determined from those actually found in the contaminated organism. The isolation of the components to be determined from the body fluids is usually effected by extraction, *e.g.*, with dichloromethane, diethyl ether, *n*-hexane or ethyl acetate[104–111]. The extract obtained may subsequently be purified in columns filled with Sep-Pak $C_{18}$ or on plates coated with silica gel and then extracted again[112–115].

In the analysis of biological samples, the headspace technique may also be used. It has been applied, for instance, for the isolation of hydrogen cyanide from blood[116,117] and of mustard gas from urine[118,119]. Chemical derivatization of the sample components may also be used for the isolation of chemical warfare agents from biological matter[120]. If tissue is the sample material, it is extracted, after homogenizing, with water and the aqueous extract is concentrated[121,122].

A scheme of the procedure for the chromatographic analysis of various materials contaminated with chemical warfare agents is given in Fig. 1. The choice of a suitable chromatographic technique depends on several factors: availability of apparatus, professional training of personnel, conditions under which the analysis is to be carried out, time allowed for the analysis and purpose for which the results are designed (qualitative, semi-quantitative, quantitative) and their accuracy. Consideration of these factors, and the number of publications involving different techniques, indicate that today the most useful for the analysis of chemical warfare agents is GC, TLC and particularly HPLC being of lesser importance.

## 3. ANALYSIS OF CHEMICAL WARFARE AGENTS BY THIN-LAYER CHROMATOGRAPHY

### *3.1. General*

TLC is widely used in many analyses, including routine qualitative, semi-quantitative and quantitative applications. The present state of art of TLC, which has been very well described by Geiss[123], shows development along three main lines: devel-

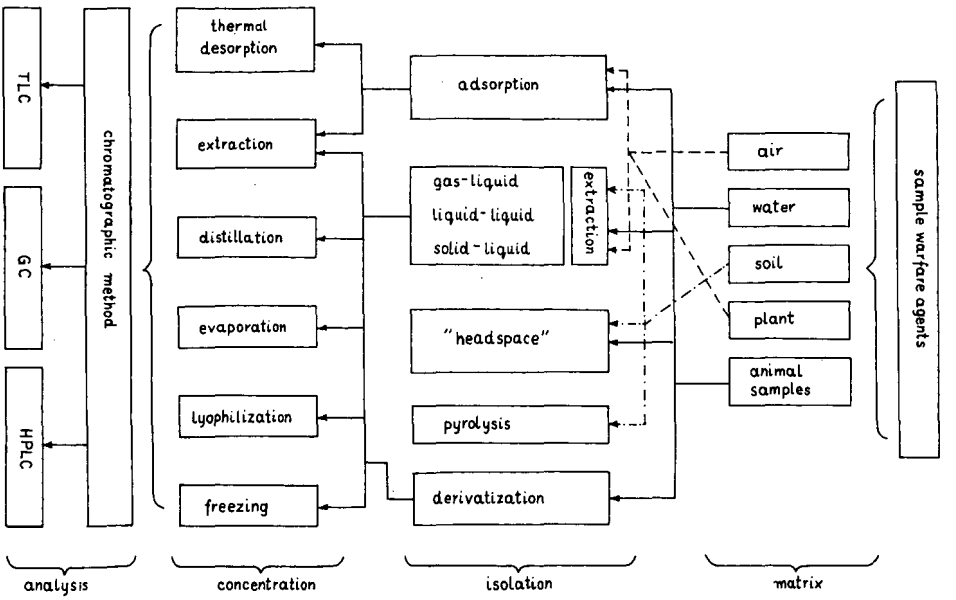



Fig. 1. Scheme for sample preparation of different materials contaminated with chemical warfare agents.

opment of new chromatographic chambers distinguished by their higher separating efficiency and shorter times of developing *e.g.*, overpressured TLC (OPTLC)[124–130] or the Soczewinski chamber[131,132], seeking new materials for the thin layers in high-performance TLC (HPTLC)[133,134]; and seeking new methods for detection and better interpretation of chromatograms[135–137].

The high selectivity, high detectability and reliability of analysis under fairly simple conditions contribute to the effective use of TLC for the detection of most chemical warfare agents in both fixed and mobile (field) laboratories. If the chemical warfare agents are to be determined in a sample of unknown provenance, and it is necessary to repeat many times the separation and identification of the sample components under various conditions, then the TLC method is very useful.

Application of TLC for military purposes, including analytical procedures for chemical warfare agents, has been recommended by many workers[6,7,138,139]. In a report prepared by the Ministry of Foreign Affairs in Finland, the use of TLC for detecting chemical warfare agents on the battlefield is recommended and it is instructed that it be included in the outfit of a mobile laboratory[6]. TLC is one of the basic methods used in some armies for the detection of chemical warefare agents under field conditions.

The adsorbent used as the stationary phase has less influence than the mobile phase on the course and results of analysis carried out by TLC[140]. Most commonly silica gel is used in the analysis of chemical warfare agents. Aluminium oxide is used to a much lesser extent, and reports of the use of cellulose or polyamide are exceptional. The selection of a suitable developing system with an adequate eluting capacity ensures the required separation of a sample mixture[141].

The basis for the identification of a chemical warfare agent is the location of its

spot on the chromatogram, which can be expressed in quantitative terms by the $R_F$ value. Accurate identification in this way requires a highly selective developing system and accurate observance of the prescribed conditions regarding the kind of chromatographic chamber, plate, developing system and temperature. However, if we take advantage of the additional information as to the colour of the spot, which is revealed by spraying the plate with a selective detection reagent, then a proper identification may be possible even without accurate observation of the specified conditions. The limit of detection of chemical warfare agents by TLC and common colour reactions is $10^{-6}$–$10^{-9}$ of the compound in the spot.

*3.2. Organophosphorus compounds*

Organophosphorus chemical warfare agents were discovered shortly before World War II and have been intensively investigated ever since. The earliest group of compounds obtained are denoted by the letter G; those obtained later are more toxic and bear the symbol V (VX, VN). All organophosphorus agents are lethal, their action consisting in the inhibitive blocking of cholinesterase. If we take advantage of this enzymatic reaction for visualizing thin-layer chromatograms, very good detection of organophosphorus compounds is achieved[142,143]. McKinley and co-workers[144,145] were the first to apply the enzyme inhibition reaction in chromatographic analysis in the early 1960s and subsequently the same group[146,147] created the foundations of modern enzymatic analysis. Ever since, many papers have been published[148–151], and also surveys[152], on the determination of organophosphorus pesticides and other enzyme inhibitors. The detection limit of the inhibitor being analysed depends on its origin and on the conditions and time of storage of the enzyme, and lies in the range $10^{-9}$–$10^{-12}$ g of the inhibitor in the spot[99,138,151].

An example of the application of the enzymatic method to the detection and identification of organophosphorus agents was described by Stachlewska-Wróblowa[138]. She sprayed the plate with an aqueous solution of the enzyme and then with a mixture of 2-naphthol acetate and diazotized *o*-dianisidine in aqueous alcohol. At the sites where organophosphorus compounds were present white spots appeared on the intensely violet background of the plate. This method of detection made it possible to detect 10 ng of the substance in the spot. With $VN_1$ two spots were obtained, which was ascribed to the presence of two isomers, thiol and thionic. She also used indoxyl acetate and its derivatives as detection reagents. As a result of enzymatic hydrolysis, fluorescent indoxyl was formed at the sites where the enzyme inhibitor was absent. Dark, non-fluorescent spots on a bluish green background visible under UV light made it possible to detect sarin and soman in amount of 5 ng per spot.

The presence of other inhibitors of enzymes in samples of chemical warfare agents complicates TLC with enzymatic detection, although it is still possible, which was shown by distinguishing organophosphorus pesticides and carbamates blocking cholinesterase from warfare agents[153]. Five selective detection reactions were applied, which made possible the identification of ten insecticides, soman and VX. The total time of analysis did not exceed 30 min.

Analysis of the chromatograms of organophosphorus compounds of the VX type containing a ternary nitrogen atom in the molecule and separated on silica gel plates showed that these compounds usually remain at the start owing to the formation of salts on the slightly acidic surface of the adsorbent. It was possible to eliminate

this effect by adding to the mobile phase a small amount of a base, *e.g.*, diethylamine[154]. It is not desirable to adopt this approach when analysing complex mixtures of chemical warfare agents as some of them, *e.g.*, sarin, react with the amine to give amides.

DFP can be well detected without resort to the enzymatic reaction. Jacobson and Patchornik[155] studied the possibility of detecting DFP by using coloured nitrophenols and nitrothiophenols, which are electrophilic reagents. The highest detectability of DFP was achieved when 2,4-dinitrothiophenol (DNPS) and 2,4,6-trinitrothiophenol (TNPS) were used. The brown (DNPS) or orange (TNPS) spot was visible for several minutes, after which it became decolorized. The spot became coloured again after it was sprayed with a solution of sodium hydroxide. The detection reaction was tested on various plates, *e.g.*, with silica gel, cellulose or polyamide, and in all instances similar results were obtained. The high detectability (1–2 nmol in the spot) was stated to be due to the hydrolysis of DFP and formation of fluoride ion. Stachlewska-Wróblowa[156] described the analysis of a mixture of twelve compounds (organophosphorus, necrotic and irritant compounds). The detection of organophosphorus chemical warfare agents by the enzymatic method was hindered by the appearance of yellow spots due to CS and chloroacetophenone and, when iodine activation was applied, also spots due to adamsite appeared. In order to achieve complete identification of the particular compounds, analysis was conducted in two chromatographic systems. In the first, the mobile phase was *n*-hexane – dioxane – pyridine. The spots were detected using the enzymatic reaction (spots of sarin, soman, tabun, $VN_1$, CS and CN appeared) and Rhodamine B and Tollens reagent (spots of HD, CA, CS, CN and DM appeared). The separation of the irritants (CS, CN and DM) from the organophosphorus compounds (sarin, tabun) was carried out with a mobile phase consisting of dichloroethane and ethyl acetate. The spots were revealed by the enzymatic method. Preliminary spraying of the plate with a solution of iodine in chloroform improved the contours of the spots and made possible the detection of adamsite.

A mixture containing sarin, soman and VX with six other chemical warfare agents was chromatographed in normal and pressure chromatographic chambers. The chromatograms obtained were similar, although the $R_F$ values using the pressure chamber were higher. For example, the $R_F$ value for soman in the pressure chamber was 0.7 and in the normal saturated chamber 0.38. The chromatogram development time in the pressure chamber was much shorter than that in the normal chamber.

### *3.3. Vesicant compounds*

Vesicant (blistering) warfare agents act locally on the body surface giving symptoms similar to scorches with necrosis of the tissue. They also exert a toxic effect on the whole organism which may lead to death. Among necrotic compounds, mustard gas [bis (2-chloroethyl) sulphide] is the most important; it was used first during World War I, so its chemical analysis is well developed.

Today many types of sulphur and nitrogen yperites are known. Their analysis, especially in multi-component mixtures, by conventional chemical methods is difficult, whereas it is fairly easy by chromatographic methods. A mixture of sulphur and nitrogen yperites was separated by Sass and Stutz[107]. They used as the group reagent 4-(4′-nitrobenzyl)pyridine, which gave a blue spot with all yperites. The compounds

belonging to the sulphur or nitrogen yperite group with similar $R_F$ values were detected with various agents. This made it possible to distinguish 1,2-bis(2-chloroethyl) thioloethane (Q) from 2,2',2''-trichlorotriethylamine (HN-3), for which the $R_F$ values were 0.68 and 0.66, respectively. The detection limit for Q was two orders of magnitude lower than that for HN-3. In general, yperites were determined at the microgram level.

Mustard gas was also determined in a mixture containing organophosphorus and/or organochlorine insecticides[158]. The identification of mustard gas was possible owing to the use of a solvent in which mustard gas has a high $R_F$ value and the remaining components a low value or by detection of the spot with a selective reagent, *e.g.*, iodoplatinate ($PtI_6^{2-}$). The sensitivity of the reaction allowed the detection of mustard gas at the submicrogram level.

Munavalli and Pannella[159] analysed mustard gas and its metabolites in biological fluids, testing fifteen developing systems. For detection a solution of potassium permanganate and sodium carbonate was used, which yielded yellow spots on a pink background. The spots were stable for many hours. It was also found that the chromatograms can be detected with a solution of 4-(4'-nitrobenzyl)pyridine in acetone.

Heating of the plate and its exposure to the action of ammonia vapour developed blue spots of mustard gas. The latter method allows mustard gas to be detected in an amount of about 0.056 $\mu$g in 1 $cm^3$ of solution.

The above methods of analysing yperites by TLC with the use of chemical reactions for detection make it possible to detect and determine yperites present in microgram amounts. Similar possibilities exist when a biochemical reaction is used for the detection of mustard gas on the chromatograms[160]. Mustard gas may be determined quantitatively on the thin-layer chromatogram several hours after development. By measuring the radioactivity of mustard gas labelled with $^{35}S$ it was shown that the losses of mustard gas in chromatograms stored for 24 h do not exceed 5%[161].

A survey of the applications of paper, thin-layer and gas chromatographic methods for detecting alkylating agents, and also sulphur and nitrogen yperites, was published by Fishbein and Falk[162].

Vesicant warfare agents also include organic arsines. However, in view of their lesser importance, many fewer examples of their analysis have been reported. Stachlewska-Wróblowa analysed primary, secondary and tertiary organic arsines[163,164].

### *3.4. Irritants*

The irritant agents include lachrymators and sternites. These agents are not lethal but by acting on the eyes (lachrymators) and on the respiratory tract (sternites) they hinder normal functioning. This group of agents include substances that differ considerably in chemical structure, which makes their analysis fairly difficult. This is due to, among other things, the large differences in their polarity. Ludemann *et al.*[165] drew attention to this fact when analysing irritant agents and the contaminants commonly present in them. They described the use of plates with different adsorbents, different developing systems and various detection agents in the analysis of bromobenzyl cyanide, *o*-chlorobenzalmalononitrile, chloroacetophenone and diphenylaminochloroarsine. Under optimum conditions it was possible to detect 1 $\mu$g or even less of the agent. A similar detectability of irritants was also achieved by other workers[138,166,167].

TABLE 2

EXAMPLES OF ANALYSIS OF CHEMICAL WARFARE AGENTS BY TLC

| *Chemical warfare agent* | *TLC plate* | *Mobile phase* | *Detection reagent* | $R_F$ | *Ref.* |
|---|---|---|---|---|---|
| *Organophosphorus compounds* | | | | | |
| GA | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–β-naphtol acetate + *o*-dianisidine | 0.48 | 138, 156 |
| | | *n*-Hexane–pyridine (4:1) | | 0.36 | 156 |
| | | Dichloroethane–ethyl acetate (7:3) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.54 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.55 | 156 |
| | Silica gel | Dichloromethane | | 0.05 | 6 |
| | | Acetone | | 0.60 | 6 |
| | | *n*-Heptane–acetone (3:2) | | 0.00 | 6 |
| | | Chloroform–methanol (4:1) | | 0.73 | 6 |
| | | *n*-Hexane–acetone–dichloromethane (7:2:1) | 5% NaOH + 1% $H_2O_2$–cobalt(III) chloride solution | 0.26 | 154 |
| GB | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.57 | 138, 156 |
| | | *n*-Hexane–pyridine (4:1) | | 0.48 | 156 |
| | | Dichloroethane–ethyl acetate (7:3) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.44 | 156 |

*(Continued on p. 308)*

TABLE 2 (*continued*)

| *Chemical warfare agent* | *TLC plate* | *Mobile phase* | *Detection reagent* | $R_F$ | *Ref.* |
|---|---|---|---|---|---|
| | Silica gel | *n*-Heptane–acetone (3:2) | | 0.17 | 6 |
| | | Chloroform–methanol (4:1) | | 0.76 | 6 |
| | | Dichloromethane | | 0.05 | 6 |
| | | Acetone | | 0.63 | 6 |
| | | *n*-Hexane–acetone–dichloromethane (7:2:1) | | 0.27 | 154 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | ChE solution–IBCh solution + Michler's hydrol | 0.48 | 157 |
| GD | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.67 | 138, 156 |
| | | Dichloroethane–ethyl acetate (9:1) | Aldehyde reagent | 0.42 | 153 |
| | | Ethyl acetate | Aldehyde reagent | 0.95 | 153 |
| | | Dichloroethane | Aldehyde reagent | 0.14 | 153 |
| | | *n*-Hexane–pyridine (4:1) | | 0.59 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.70 | 156 |
| | | Dichloroethane–ethyl acetate (7:3) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.62 | 156 |
| | Silica gel | *n*-Hexane–acetone–dichloromethane (7:2:1) | 5% NaOH + 1% $H_2O_2$–cobalt(III) chloride solution | 0.41 | 154 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | ChE solution–IBCh solution + Michler's hydrol | 0.70 | 157 |

| | | | | | |
|---|---|---|---|---|---|
| DFP | Silica gel | Dichloromethane | | 0.10 | 6 |
| | | *n*-Heptane–acetone (3:2) | | 0.33 | 6 |
| | | Acetone | | 0.67 | 6 |
| | | Chloroform–methanol (4:1) | | 0.75 | 6 |
| | | *n*-Hexane–acetone–dichloromethane (7:2:1) | 5% NaOH + 1% $H_2O_2$–cobalt (III) chloride solution | 0.50 | 154 |
| VX | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–$\beta$-naphthol acetate + *o*-dianisidine or 0.5% iodine solution | 0.05<br>0.18 | 138,<br>156 |
| | | Dichloroethane–ethyl acetate (9:1) | 0.5% Iodine solution or 0.5% $KMnO_4$ solution | <0.06 | 153 |
| | | Ethyl acetate | 0.5% Iodine solution or 0.5% $KMnO_4$ solution | <0.15 | 153 |
| | | Dichloroethane | 0.5% Iodine solution or 0.5% $KMnO_4$ solution | <0.08 | 153 |
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent–UV | 0.00 | 156 |
| | | *n*-Hexane–pyridine (4:1) | | 0.10 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | Rhodamine B–Tollens reagent–UV or ChE solution–$\beta$-naphthol acetate + *o*-dianisidine | 0.21 | 156 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (4:1) | ChE solution–IBCh solution + Michler's hydrol | 0.03 | 157 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | 0.5% Iodine solution | 0.64 | 156 |
| *Vesicant compounds* | | | | | |
| HD | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | Rhodamine B–Tollens reagent–UV | 0.71 | 138,<br>156 |
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent–UV | 0.72 | 156 |

*(Continued on p. 310)*

TABLE 2 (*continued*)

| *Chemical warfare agent* | *TLC plate* | *Mobile phase* | *Detection reagent* | $R_F$ | *Ref.* |
|---|---|---|---|---|---|
| | | *n*-Hexane–acetone–pyridine (7:2:1) | Rhodamine B–Tollens reagent–UV | 0.75 | 156 |
| | Silica gel | *n*-Hexane–acetone (7:3) | | 0.57 | 6 |
| | | Dichloromethane | | 0.65 | 6 |
| | | Chloroform–methanol (4:1) | | 0.72 | 6 |
| | | Dichloromethane | 4-(4′-Nitrobenzyl)pyridine–5% sodium perchlorate–piperidine or *o*-dianisidine solution–copper(II) acetate–96% $H_2SO_4$ | 0.77 | 107 |
| | Silica gel 60 $F_{254}$ | Dichloromethane | Palladium chloride solution | 0.77 | 114 |
| | Silica gel (Merck 5721) | Benzene–chloroform (1:1) | | 0.79 | 156 |
| | | Benzene–acetic acid (9:1) | | 0.88 | 156 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | Michler's ketone + mercury(I) chloride solution | 0.67 | 157 |
| | Silica gel | Dichloromethane–*n*-hexane (1:1) | Iodoplatinate solution $[PtI_6]^{2-}$ or Michler's ketone + mercury bromide or silver nitrate–UV or triphenylmethane solution + silver nitrate or selenic acid | >0.80 | 158 |
| | Silica gel GF | Chloroform–acetone (5:4) | 4-(4′-Nitrobenzyl)pyridine or 1% $KMnO_4$ solution | 0.89 | 159 |
| | | Acetonitrile–ethanol (10:1) | 4-(4′-Nitrobenzyl)pyridine or 1% $KMnO_4$ solution | 0.92 | 159 |
| | | Acetonitrile | 4-(4′-Nitrobenzyl)pyridine or 1% $KMnO_4$ solution | 0.96 | 159 |

| | | | | | |
|---|---|---|---|---|---|
| | Silica gel GF or HPTLC GHLF or Whatman LHP-KF | Chloroform–methanol (10:1) | 4-(4′-Nitrobenzyl)pyridine or 1% $KMnO_4$ solution | 0.94 | 159 |
| | | Chloroform–acetonitrile (5:1) | 4-(4′-Nitrobenzyl)pyridine | 0.95 | 159 |
| | Silica gel G | Chloroform–acetone (5:4) | Radiation detector | 0.80 | 161 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | Tollens reagent–UV | 0.87 | 156 |
| HN-3 | Silica gel | *n*-Hexane–acetone (7:3) | | 0.50 | 6 |
| | | Dichloromethane | | 0.55 | 6 |
| | | Chloroform–methanol (4:1) | | 0.78 | 6 |
| | Silica gel G | Dichloromethane | 4-(4′-Nitrobenzyl)pyridine–5% sodium perchlorate–piperidine or *o*-dianisidine solution–copper(II) acetate–96% $H_2SO_4$ | 0.66 | 107 |
| L | Silica gel | *n*-Hexane–acetone (7:3) | | 0.00 | 6 |
| | | Dichloromethane | | 0.00 | 6 |
| | | Chloroform–methanol (4:1) | | 0.58 | 6 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | Michler's thioketone–formalin + 96% $H_2SO_4$ | 0.00 | 157 |
| | Silica gel (Merck 5553 or Merck 5748) | Benzene–acetic acid–2-propanol (17:1:2) | Bromocresol purple solution–25% ammonia or dithizone–acetic acid or 0.1% iodine solution or Michler's thioketone solution | 0.19 | 163, 164 |
| *Irritant compounds* | | | | | |
| CA | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | Rhodamine B–Tollens reagent–UV | 0.59 | 138, 156 |

*(Continued on p. 312)*

TABLE 2 (*continued*)

| *Chemical warfare agent* | *TLC plate* | *Mobile phase* | *Detection reagent* | $R_F$ | *Ref.* |
|---|---|---|---|---|---|
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent–UV | 0.60 | 156 |
| | Silica gel | Toluene–dichloroethane (1:1) | Thiocarbamide–*o*-dianisidine or Michler's thioketone solution | 0.51 | 138 |
| | Silica gel (Merck 5721) | Benzene–chloroform (1:1) | | 0.67 | 156 |
| | | Benzene–acetic acid (9:1) | | 0.72 | 156 |
| | Silica gel G | Benzene | 4-(4'-Nitrobenzyl)pyridine–5% sodium perchlorate–piperidine | 0.52 | 165 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | Tollens reagent–UV | 0.87 | 156 |
| | Acid alumina gel | Dichloromethane–benzene (1:3) | 4-(4'-Nitrobenzyl)pyridine–5% sodium perchlorate–piperidine or iodine | 0.58 | 165 |
| | | Chloroform–benzene (1:19) | Quinone solution or quinone + 5% NaOH or 4-(4'-nitrobenzyl)pyridine–5% sodium perchlorate–piperidine | 0.65 | 165 |
| CN | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–$\beta$-naphthol acetate + *o*-dianisidine or Rhodamine B–Tollens reagent–UV | 0.57 | 138, 156 |
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent–UV | 0.44 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | Rhodamine B–Tollens reagent–UV or ChE solution –$\beta$-naphthol acetate + *o*-dianisidine or benzofurazan oxide + 1 *M* NaOH or thiocarbamide solution + *o*-dianisidine | 0.63 | 156 |

| | | | | | |
|---|---|---|---|---|---|
| | | Dichloroethane–ethyl acetate (7:3) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.78 | 156 |
| | Silica gel | *n*-Hexane–acetone (7:3) | | 0.47 | 6 |
| | | Dichloromethane | | 0.57 | 6 |
| | | Toluene–dichloroethane (1:1) | Thiocarbamide–*o*-dianisidine or Michler's thioketone solution | 0.36 | 138 |
| | Silica gel (Merck 5721) | Benzene–chloroform (1:1) | | 0.48 | 156 |
| | | Benzene–acetic acid (9:1) | | 0.50 | 156 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | *m*-Dinitrobenzene–5 *M* KOH | 0.69 | 157 |
| | Silica gel (Merck 5553) | Chloroform | *m*-Dinitrobenzene + benzofurazan oxide–5 *M* KOH or $KMnO_4$ solution | 0.41 | 168 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | Tollens reagent–UV | 0.87 | 156 |
| | Acid alumina gel | Dichloromethane–benzene (1:3) | Iodine or 4-(4′-nitrobenzyl)pyridine–5% sodium perchlorate–piperidine | 0.45 | 165 |
| | | Chloroform–benzene (1:19) | Quinone solution–5% NaOH or 4-(4′-nitrobenzyl) pyridine–5% sodium perchlorate–piperidine | 0.50 | 165 |
| CR | Silica gel | Dichloromethane | | 0.10 | 6 |
| | | *n*-Hexane–acetone (7:3) | | 0.37 | 6 |
| | Silica gel (Merck 5553) | Chloroform | Dragendorff's reagent or $KMnO_4$ solution | 0.12 | 168 |
| CS | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | ChE solution–β-naphthol acetate + *o*-dianisidine or Rhodamine B–Tollens reagent–UV | 0.57 | 138, 156 |

*(Continued on p. 314)*

TABLE 2 (*continued*)

| *Chemical warfare agent* | *TLC plate* | *Mobile phase* | *Detection reagent* | $R_F$ | *Ref.* |
|---|---|---|---|---|---|
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent–UV | 0.48 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | Rhodamine B–UV or ChE solution–β-naphthol acetate + *o*-dianisidine or benzofurazan oxide + 1*M* NaOH | 0.63 | 156 |
| | | Dichloroethane–ethyl acetate (7:3) | ChE solution–β-naphthol acetate + *o*-dianisidine | 0.78 | 156 |
| | Silica gel | *n*-Hexane –acetone (7:3) | | 0.43 | 6 |
| | | Dichloromethane | | 0.60 | 6 |
| | | Toluene–dichloroethane (1:1) | Thiocarbamide–*o*-dianisidine or Michler's thioketone solution | 0.43 | 138 |
| | Silica gel (Merck 5721) | Benzene–chloroform (1:1) | | 0.53 | 156 |
| | | Benzene–acetic acid (9:1) | | 0.56 | 156 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | *m*-Dinitrobenzene–5 *M* KOH | 0.67 | 157 |
| | Silica gel (Merck 5721) | Chloroform | *m*-Dinitrobenzene + benzofurazan oxide–5 *M* KOH or $KMnO_4$ solution | 0.45 | 168 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | Tollens reagent–UV | 0.87 | 156 |
| | Acid alumina gel | Dichloromethane–benzene (1:3) | Iodine or 4-(4'nitrobenzyl)pyridine–5% sodium perchlorate–piperidine | 0.53 | 165 |

| | | | | | |
|---|---|---|---|---|---|
| | | Chloroform–benzene (1:19) | Quinone solution or quinone + 5% NaOH or 4-(4′ nitrobenzyl)pyridine–5% sodium perchlorate–piperidine | 0.55 | 165 |
| DM | Silica gel 60–Kieselguhr $F_{254}$ (Merck 5567) | *n*-Hexane–pyridine–dioxane (7:2:1) | Rhodamine B–Tollens reagent or 0.5% iodine solution | 0.24 | 138, 156 |
| | | Benzene–*n*-hexane (3:1) | Rhodamine B–Tollens reagent | 0.00 | 156 |
| | | *n*-Hexane–acetone–pyridine (7:1:2) | Rhodamine B–UV | 0.28 | 156 |
| | | Dichloroethane–ethyl acetate (7:3) | Iodine solution–ChE solution–β-naphthol acetate + *o*-dianisidine | 0.29 | 156 |
| | Silica gel (Merck 5721) | Dichloromethane–2-propanol–ethyl acetate (2:1:1) | | 0.79 | 156 |
| | Silica gel (Merck 5562) | Acetone–carbon tetrachloride (1:4) | Michler's thioketone–formalin + 96% $H_2SO_4$ | 0.37 | 157 |
| | Silica gel (Merck 5553 or 5748) | Benzene–acetic acid–2-propanol (17:1:2) | Bromocresol purple solution–25% ammonia or dithizone–acetic acid or 0.1% iodine solution or Michler's thioketone solution | 0.47 | 163, 164 |
| | Basic silica gel G | Ethyl acetate–methanol (1:9) | *o*-Dianisidine–copper(II) acetate–50% $H_2SO_4$ | 0.23 | 165 |
| | Silica gel G | Acetone–chloroform (1:4) | *o*-Dianisidine–copper(II) acetate–50% $H_2SO_4$ | 0.35 | 165 |
| | Alumina gel (Merck 5575) | Benzene–acetic acid (4:1) | Iodine solution or Tollens reagent–UV | 0.72 | 156 |
| | Acid alumina gel | Chloroform–benzene (1:19) | *o*-Dianisidine–copper(II) acetate–50% $H_2SO_4$ | 0.00 | 165 |
| *Psychotoxic compound* | | | | | |
| BZ | Silica gel | Acetone–carbon tetrachloride (1:4) | Formalin + 96% $H_2SO_4$ | 0.06 | 157 |

Dibenzo[*b,f*]-1,4-oxazepine (CR) was analysed in the presence of *o*-chlorobenzalmalononitrile and chloroacetophenone by Rosłonek *et al.*[168]. Good separations were obtained when chloroform was used as the eluent and Dragendorff reagent as a specific detection reagent for CR. The detection limit of CR was 0.2 mg/cm$^3$. The presence of other chemical warfare agents in the mixture had no effect on the elution and identification of the irritants.

In Table 2, examples are given of the analysis of chemical warfare agents by TLC.

## 4. ANALYSIS OF CHEMICAL WARFARE AGENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

### *4.1. General*

Theoretically, all chemical warfare agents analysed by TLC can also be analysed by HPLC. The practical difficulty consists, however, in the lack of a suitable detector for some of these substances. Mostly in HPLC, detectors are used in which advantage is taken of UV absorption by the compounds being detected. The use of such detectors is therefore limited to compounds that contain chromophore groups active in the UV region or to those wich can easily be converted into compounds with such groups. UV-detection allows the analysis of irritants and analogous phytotoxic compounds at the nanogram level. Most chemical warfare agents, however, do not show absorption in the UV region or the absorption is very weak, and their conversion to UV-active derivatives complicates the analysis.

Fluorescence and electrochemical detectors are also used in HPLC. A typical fluorescence detector is about 1000 times more sensitive than a UV detector. The use of such a detector is, however, limited to fluorescent compounds or compounds that can easily be converted into such. Hence fluorescence detectors find very limited application in analysis of chemical warfare agents. The applicability of electrochemical detectors in such analyses is even more restricted.

Other types of detection system not commonly used but finding application in HPLC might also be used in the analysis of chemical warfare agents and their analogues, *e.g.* flame ionization detection (FID)[169] and flame photometric detection (FPD)[170,171]. Spectroscopic methods have also been used, *e.g.*, mass spectrometry (MS)[172–175], Fourier transformation IR (FT-IR)[8] and ion-mobility spectrometry[176–179]. Good results were obtained by applying nuclear magnetic resonance (NMR)[8], and also a detection system taking advantage of the transformations of the chemical substance eluted from the column[180]. In the analysis of organophosphorus agents, detection involving the use of enzymatic reactions is particularly desirable[9–13,181]. Using this detection method it is possible to determine organophosphorus compounds at the picogram level.

In HPLC, non-polar stationary phases chemically bonded to the substrate are today chiefly used as column fillings; these are fillings for reversed-phase chromatography. They allow the analysis of complex mixtures containing, as is often the case with chemical warfare agents, compounds with different functional groups. For the separation of chemical warfare agents, standard stainless-steel columns are used, filled with, *e.g.*, LiChrosorb Hibar RP-18, Spherisorb S5 ODS-2 or Zorbax ODS. The advantages of these stationary phases are their resistance to the destructive action of

eluents of different pH, the possibility of introducing aqueous samples directly into the chromatographic column and the relatively rapid establishment of thermodynamic equilibrium of the chromatographic system.

In accordance with the general properties of HPLC, the quality of separation of chemical warfare agents depends on the composition of the eluent. In most instances good results are obtained by applying isocratic chromatography, although sometimes, especially when the mixture is very complex (organophosphorus agents in the presence of HD, CS, DM and its hydrolysis product and BZ), it is recommended to apply gradient chromatography[12,55,182]. In most commonly used types of reversed-phase chromatography the eluent usually includes water and methanol or acetonitrile.

The identification of chemical warfare agents separated in the chromatographic column can be made on the basis of the retention indices relative to a selected homologous series[183–185]. For instance, for the identification of irritants or psychotoxins, use is made of the alkyl aryl ketone and 1-*p*-(2,3-dihydroxypropyloxy)phenylalkanone homologous series[186].

*4.2. Examples*

For organophosphorus chemical warfare agents the enzymatic method of detection is the most appropriate in view of the required detectability. It takes advantage of the inhibition of the enzyme by the organophosphorus compound. The non-inhibited enzyme decomposes certain chemical compounds (substrates), *e.g.*, butyrylcholine iodide. As a result, the pH of the solution changes, producing a change in colour of an added acid–base indicator. After inhibition the enzyme has a lower ability to decompose the substrate, so the pH of the medium is less affected and the colour of the indicator changes more slowly or does not change at all. Usually only part of the eluate from the column is introduced into the reaction vessel, and the effect of the reaction is determined spectrophotometrically[55,187]. A schematic diagram and an example of enzymatic detection in HPLC are shown in Fig. 2.

One of the problems involved in enzymatic detection is the composition of the eluent. Enzymatic reactions proceed best in aqueous solutions, and certain organic solvents affect the course of these reactions very negatively. For instance, acetonitrile is less useful than methanol as a component of the eluent[55]. The results of analysis obtained also depend on the kind of enzyme used. This is shown in Fig. 3 for acetylcholinesterases obtained from an electric eel and from human serum. Acetylthiocholine iodide was used as the substrate in this reaction and 5,5-dithiobis(2-nitrobenzoic acid) as the colour reagent[55]. The detection limit achieved for sarin and soman was 10 pg and for tabun 60 pg.

The enzymatic detector not only reveals the presence in the sample of organophosphorus agents but also indicates the presence of other inhibitors of enzymes such as organophosphorus pesticides and carbamates. The analysis of chemical warfare agents in the presence of other inhibitors may be difficult if their separation is incomplete. This problem may be solved, however, by applying additionally a UV detector sensitive only to compounds posessing chromophore groups (which is the case with most pesticides).

Analysis of organophosphorus agents with the help of HPLC can also be carried out by derivatization to introduce a chromophore or fluorescent substituent into

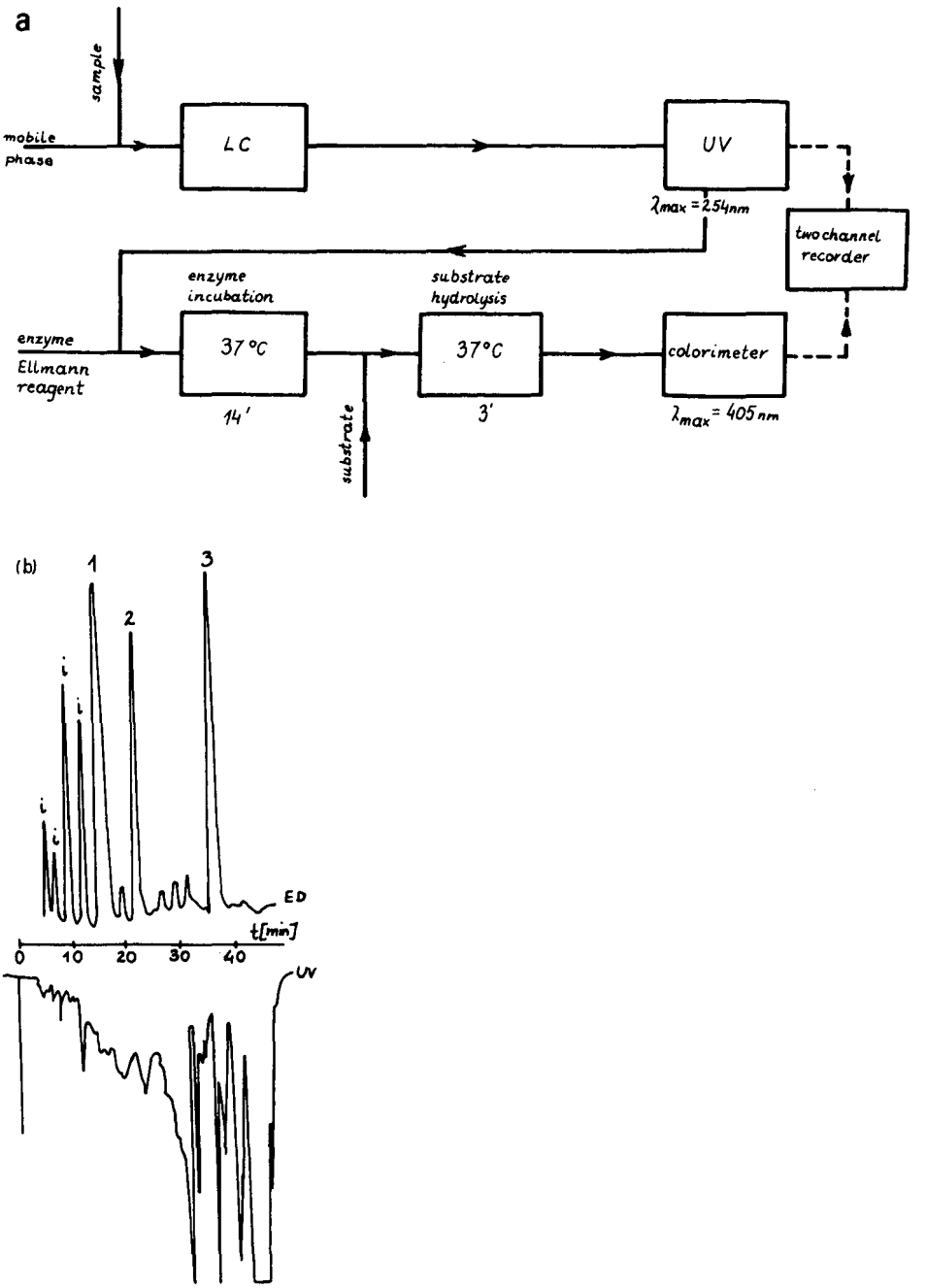



Fig. 2. (a) Scheme of the HPLC–UV enzymatic detection system for analysis of air contaminated with phosphorus chemical warfare agents and (b) the chromatogram obtained with the system. i = Impurities; 1 = sarin; 2 = tabun; 3 = soman. Conditions: 250 mm × 4.0 mm I.D. column with 5-μm LiChrosorb RP-18; linear gradient, 15–65% methanol in water in 35 min; flow-rate, 0.7 ml/min; enzymatic detection with human serum ChE[55].

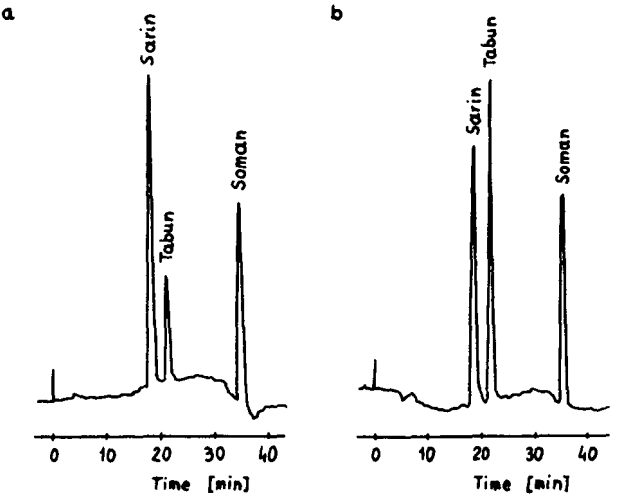



Fig. 3. Chromatogram of sarin, soman and tabun in acetone obtained by using (a) electric eel AChE and (b) human serum ChE. Conditions: 250 mm × 4.0 mm I.D. column with 5-μm LiChrosorb RP-18; linear gradient, 15–65% methanol in water in 35 min; flow-rate, 0.7 ml/min[55].

the molecule. This method was applied to identify the products of hydrolysis of organophosphorus agents after their reaction with pentafluorobenzyl bromide[8]. By applying this procedure it was possible not only to use a UV detector but sometimes also to achieve a better chromatographic separation when the detectability of the chromatographed substances was good.

By using octadecylsilane as the stationary phase, methanol–water as the mobile phase and a UV detector it was possible to obtain good results in the analysis of irritant mixtures[9–11]. For detecting particular species it is recommended to take measurements at the following wavelengths[13,186]: DM (hydrolysis products), 224, 282; CS, 220, 254, 280, 300; CN, 254 and CR, 280, 313 nm. These substances can be detected at nanogram levels, the retention times being moderate and the reproducibility of the results of analysis good.

Analysis by reversed-phase chromatography of three common irritants showed that in methanol CR tends to decompose so a special technique of mixing the solvents had to be applied[188]. The results of analysis of irritants in samples of vegetable origin are usually inferior to those obtained for other samples[189]. An example of the separation of irritants is shown in Fig. 4.

Bossle *et al.*[190] determined vesicant compounds (2-chloroethyl sulphide and the products of its decomposition) after having converted them by reaction with chloramine B sodium salt into products revealing strong absorption in the UV region. The absorption maximum was observed at 254 nm. The chromatographic separations were carried out on a column filled with Radial-Pak $C_{18}$ with water–acetone as the mobile phase. It was claimed that under these conditions it is also possible to analyse mustard gas.

Reversed-phase HPLC gives good results with organoarsenic compounds. The difficult and sometimes even impossible analysis of these compounds by GC is fairly easy using HPLC[191,192].

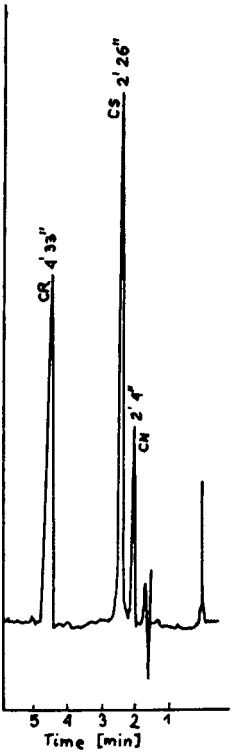



Fig. 4. Separation of CN, CR and CS by reversed-phase HPLC. Conditions: column with μBondapak $C_{18}$; mobile phase, methanol–water (7:3); flow-rate, 2 ml/min; detection, UV (280 nm); amounts, CN = 40 ng, CR = 30 ng, CS = 24 ng[188].

Among the analyses of other toxic substances, the determination of sodium fluoroacetate deserves some attention. Collins *et al.*[193] adapted the method proposed by Lam and Grushka[194] for separating fluorescent derivatives of monocarboxylic acids obtained by reaction with 4-bromomethyl-7-methoxycoumarin, using a fluorescence detector.

For the HPLC analysis of sodium fluoroacetate use was also made of the reaction with *p*-bromophenacyl bromide[121] or with *o,p*-nitrobenzyl-N,N'-diisopropylisourea[122]. This made possible the determination of sodium fluoroacetate by means of a UV detector.

The determination of phosgene by HPLC as described by Hori *et al.*[195] is worth considering. Phosgene was determined in the products of combustion of vinyl chloride by passing them through a solution of aniline.

Some chemical warfare agents may be used to analyse other organic compounds by HPLC. Someno *et al.*[196] presented a sensitive and specific method for determining the activity of two types of urokinase in human urine after their reaction with [$^3$H]DFP. The latter compound reacted selectively with urokinase to yield stable complexes. The complexes of the particular types of urokinase were separated in a chromatographic column. The eluate from the column was mixed with a liquid scintillating agent and the radioactivity was measured with a detector.

The derivatization reaction with the use of phosgene may be very useful for the HPLC separation of enantiomers of various compounds yielding oxazolidones with phosgene. A detailed description of such procedures was given in the survey by Gyllenhaal and Vessman[197].

Some examples of the analysis of chemical warfare agents by HPLC are given in Table 3.

## 5. ANALYSIS OF CHEMICAL WARFARE AGENTS BY GAS CHROMATOGRAPHY

### *5.1. General*

GC is a convenient method for analysis of complex mixtures as it allows the identification and determination of particular components. As with other organic compounds, the analysis of chemical warfare agents is possible if their vapour pressure is sufficiently high or if they can be brought into the gaseous state without decomposition or with accurately repeatable decomposition. Almost all chemical warfare agents comply with these requirements. So far, attempts to apply GC to the analysis of chemical warfare agents has failed in only a few instances which concerned chiefly arsenic compounds[10].

Initially in the analysis of chemical warfare agents by GC, packed glass or metal columns were chiefly used. For analysing compounds of high reactivity, *e.g.*, phosgene, columns made of inert materials, *e.g.*, PTFE, were used[198].

In the analysis of chemical warfare agents which are mostly polar, the choice of a suitable support, especially when trace analysis is involved, is of primary importance[199]. Hence, most often neutral silanized supports are used, chiefly Chromosorb G and W and Gas-Chrom Q and P[100,104,105,200–203]. Packed columns are being replaced to an increasing extent by capillary columns, especially the fused-silica type[6,204–207]. The availability of these columns is connected with progress in fibre-optic technology. Fused-silica capillary columns coated on the outside with polyimide or aluminium show very good mechanical strength. The inner diameter of capillary columns ranges from 0.1 to 0.75 mm. Their separating efficiency is better than that of packed columns, the time of separation is short and the peaks obtained are symmetrical.

The samples may be injected into capillary columns in different ways. One of the better solutions is that in which the sample is introduced into the column directly[208]. This method has been applied successfully in the analysis of chemical warfare agents[9,10,91] and organophosphorus pesticides[209]. A disadvantage of this method of introducing the sample into the column is the possibility of the column becoming contaminated, which may lead to a decrease in its efficiency. Despite this, the superiority of the direct method has been confirmed[210]. By direct injection of the sample into the columnn, partial or complete thermal decomposition of some compounds (*e.g.*, VX) is avoided as the necessity to evaporate the analyte substances in the injector is eliminated. Another advantage of direct injection of samples is the high accuracy of determination (2–4%) and rapid elution of the well developed peaks with good stability of the baseline[8].

A widely used procedure for injecting samples into capillary columns is that with splitting of the carrier gas stream[211]. In the analysis of chemical warfare agents, the splitting ratio of the gas stream is usually 1:10. It should be borne in mind that in

TABLE 3

EXAMPLES OF ANALYSIS OF CHEMICAL WARFARE AGENTS BY HPLC

| *Chemical warfare agent* | *Column characteristics* | *Detection* | $\lambda_{max}$ *(nm)* | *Detection limit* | *Remarks* | *Ref.* |
|---|---|---|---|---|---|---|
| BZ<br>CS<br>CN | Octadecyl silane stationary phase, mobile phase, 50% methanol–water to 100% methanol | UV | 254 | <1 ng (CN) | Separation from phenoxyacetic acids | 10 |
| GB<br>GD<br>DFP-3 | 250 mm x 4.6 mm I.D., 10-$\mu$m $C_9$ Spherisorb or 5-$\mu$m Spherisorb S 5 ODS 2; 250 mm x 4.0 mm I.D., 5-$\mu$m LiChrosorb Hibar RP-18; mobile phase, 50% methanol–water or 50% methanol–phosphate buffer | UV–enzymatic | 254 | 3.3 ng (GB)<br>1.2 ng (GD)<br>747 ng (DFP-3) | | 11 |
| DM | 250 mm x 4.6 mm I.D., 10-$\mu$m $C_9$ Spherisorb or 5-$\mu$m Spherisorb S 5 ODS 2; 250 mm x 4.0 mm I.D., 5-$\mu$m LiChrosorb Hibar RP-18; mobile phase, 50% methanol–water to 100% methanol, 50°C | UV | 282 | <1 ng | Analysis of degradation products of DM | 11 |
| BZ<br>CS<br>HD<br><br>GA<br>GB<br>GD | 250 mm x 9.4 mm I.D., 10-$\mu$m Zorbax-ODS, mobile phase, 50% methanol–water to 100% methanol, 50°C | UV<br><br><br><br>Enzymatic | 220<br>254<br>280<br>300 | | Identification and determination of chemical warfare agents in a complex mixture using single-step HPLC separation | 13 |

| | | | | | | |
|---|---|---|---|---|---|---|
| BZ<br>CN<br>CR<br>CS<br>DM | 250 mm x 4.0 mm I.D., 5-$\mu$m LiChrosorb Hibar RP-18; mobile phase, 50% methanol–water to 100% methanol, 50°C | UV | 220<br>254<br>280<br>300 | ng | Determination of retention indices | 13, 186 |
| GA<br>GB<br>GD | 250 mm x 4.0 mm I.D., 5-$\mu$m LiChrosorb RP-18; mobile phase, 15% methanol–water to 65% methanol–water | UV–enzymatic | 254 | 10 pg (GB)<br>10 pg (GD)<br>60 pg (GA) | Analysis of urban air samples | 55, 181 |
| $FCH_2COONa$ | 10-$\mu$m octadecylsilane stationary phase | UV | 254<br>280 | 1–50 ppm | Determination in canine gastric content after derivatization with *o,p*-nitrobenzyl-N,N′-diisopropylisourea | 122 |
| CN<br>CR<br>CS | $\mu$Bondapak $C_{18}$; mobile phase, ethanol–water (7:3) | UV | 254<br>280<br>313 | < 1 ng | Trace-level detection in the range 1–10 ng | 188 |
| $FCH_2COOH$ | 250 mm x 4.0 mm I.D., Lichrosorb RP-8; mobile phase, acetonitrile–water (1:2) or ethyl acetate–acetonitrile–water (9:2:22) | FD | 360 ex.<br>400 em.<br>410 em. | 0.2 ng per 100 ml | Determination in poison baits; separation of $C_1$–$C_3$ carboxylic acids | 193 |
| CG | 500 mm x 4.0 mm I.D., divinylbenzene–styrene copolymer stationary phase; mobile phase, methanol–water (9:1) | UV | 254 | 0.55 ppm | Determination in combustion gases of vinyl chloride monomer after derivatization in aniline solution | 195 |

the analysis of some organosphosphorus agents (*e.g.*, soman), strong adsorption on the active surface of the glass injector occurs. This limits significantly the detectability of the compounds being determined[9]. Hydrogen cyanide and phosgene also are strongly adsorbed or undergo decomposition, which results in additional ghost peaks on the chromatogram. These unfavourable effects are eliminated by periodic cleaning, acid deactivation or silanization of the injection system.

When using GC for the analysis of chemical warfare agents, a universal stationary phase is sought that permits the effective separation of the greatest possible number of these compounds. In the report of the Finnish Ministry of Foreign Affairs[10], it is stated that among about a dozen stationary phases tested, SE-52 and OV-1 show properties nearest to those required. These phases have high thermal stability and do not react with the chemical warfare agents. Among other phases suitable for the separation of psychotoxic agents, OV-210, Emulphor ON-870, Triton X-305, Silar 10C and FFAP are recommended[22]. SE-54, DB-5 and FFAP are recommended for use in the analysis of organophosphorus compounds, vesicants and irritants[8,13]. OV-1701 was used for analysing DFP and SE-54 for hydrogen cyanide, cyanogen chloride and phosgene[10].

Four stationary phases, DB-1, DB-5, DB-1701 and DB WAX, in the order of increasing polarity, were tested and it was found that the first three may be used for the simultaneous chromatography of most chemical warfare agents and similar compounds[91]. An example of the separation of mixture of such compounds is shown in Fig. 5.

The principal aim of the analysis of chemical warfare agents is the identification of an unknown toxic substance in the sample. This is usually done by comparing the retention indices of the substance being analysed those of a standard measured in at

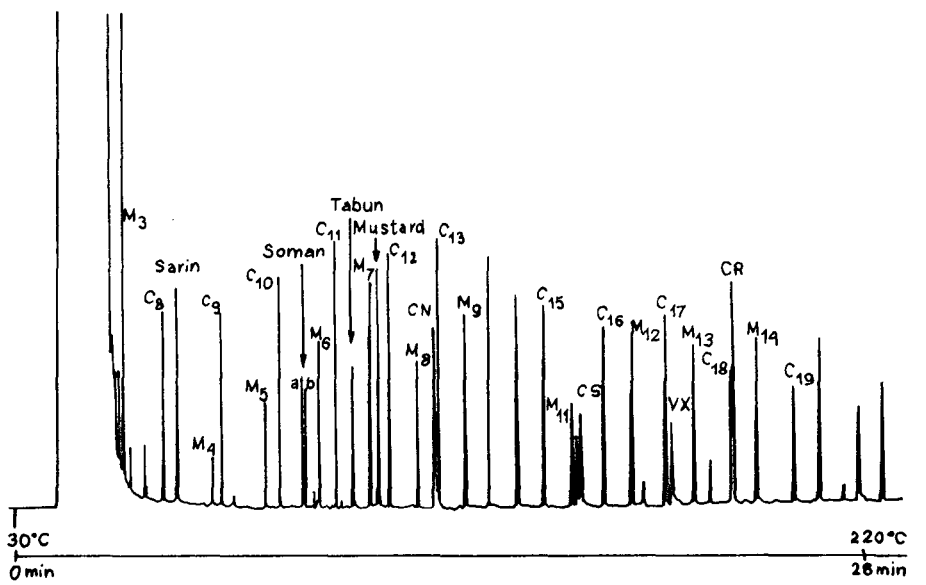



Fig. 5. Separation of chemical warfare agents and the C and M standard series mixture by GC with temperature programming. Conditions: 30 m × 0.33 mm I.D. fused-silica capillary column with 0.25-$\mu$m film of DB-5; carrier gas, helium at a flow-rate of 2 ml/min; detection, FID[13].

least two colums filled with stationary phases of different polarity. The retention indices relative to the *n*-alkane homologous series under isothermal conditions are calculated from the Kováts[212] equation and, if temperature programming is applied, form the Van den Dool and Kratz equation[213]. Usually FID or TCD is used in such instances. They allow the detection of all chemical warfare agents being separated but their sensitivities and selectivities are relatively low.

The application of a selective detector may facilitate considerably the identification of the substances being analysed. Many different selective detectors have been used in the analysis of chemical warfare agents. They may serve for detecting trace amounts of agents that contain in their molecules elements to which these detectors are particularly sensitive. Such detection methods include, electron-capture detection (ECD) for compounds containing halogens, FPD for the detection of compounds containing sulphur and phosphorus, nitrogen–phosphorus-specific detection (NPD) for compounds containing nitrogen and phosphorus, alkali flame ionization detection (AFID) and alkali thermionic detection (ATD) for organophosphorus compounds. Photoionization detection (PID) for compounds containing sulphur is gaining in importance[214,215]. Specific detectors, designed for detecting certain compounds, *e.g.*, hydrogen cyanide[216], lewisite and mustard gas[217,218], deserve mention.

Sometimes two detection methods are combined, *e.g.*, FID–AFID, FID–ECD, FID–FPD, ECD–AFID. Such systems facilitate the identification of compounds separated in one or two identical chromatographic columns[9,54,64].

Chromatographed chemical warfare agents can also be identified by confirming the presence of the compounds being detected with the use of other instrumental methods, *e.g.*, IR, NMR or MS[8,219–227]. The preferred method is combine the gas chromatograph with a mass spectrometer. Such devices are very useful for the rapid analysis of trace amounts of toxic compounds present in complex samples[220–227]. The mass spectra recorded for the components of the sample are compared with those contained in a computer memory and on this basis the particular substances are rapidly and reliably identified. The sensitivities of these devices are very good; it is possible to detect organophosphorus agents at the level of $10^{-12}$–$10^{-13}$ g[228].

The chromatographed chemical warfare agents may be identified by a technique known as retention spectrometry[13,229]. The retention spectrometer consists of several capillary columns of equal dimensions filled with stationary phases of varying polarity. After injection, the sample is divided into equal parts, each of which is directed to a different column. The same substance, after having passed through the different columns, reaches the detector, common for all columns, at different times. The registered peaks give a characteristic retention spectrum which constitutes a basis for identification of the compound of interest. This described parallel-column arrangement of retention spectrometry is used for analysing less complex samples. Samples of greater complexity are analysed by the in-series modification of retention spectrometry, in which case the sample is preliminarily separated in a conventional chromatographic column and only then is the isolated component to be determined passed to the retention spectrometer. The set-up includes two types of detection (*e.g.*, FID–TID or ECD–TID), one at the outlet of the conventional chromatographic column and the other at the outlet of the retention spectrometer. A diagram of the in-series arrangement of the retention spectrometer is shown in Fig. 6.

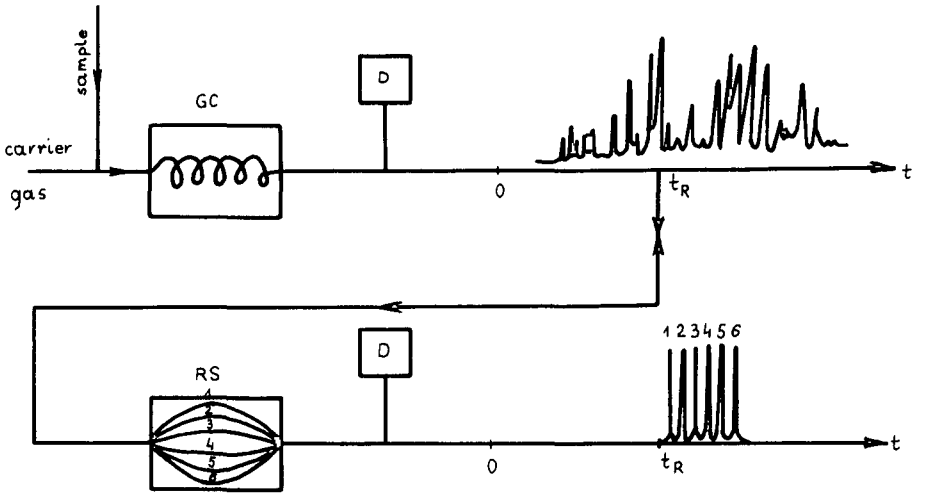



Fig. 6. Trace analysis of chemical warfare agents in complex environmental samples by in-series retention spectrometry[13].

Organophosphorus, vesicant and irritant chemical warfare agents were analysed by using a retention spectrometer including six capillary columns filled with SE-30, SE-52, SE-54, OV-1701, Carbowax 20M and OV-351[13,229].

*5.2. Organophosphorus compounds*

The earliest report on the analysis of organophosphorus chemical warfare agents appeared in 1963[230], on the analysis of sarin and its contaminants. In the course of chromatography, ghost peaks were observed whose presence was ascribed to the formation of products of sarin conversion. TCD used initially in the analysis of sarin allowed the determination of the latter at the ppm level, which was unsatisfactory in view of its high toxicity. It was only after Brody and Chaney[231] in 1966 developed a flame photometric detection system specific for phosphorus- or sulphur-containing compounds (FPD-PS) that analysis at the subnanogram level became possible[231].

Of the numerous phases on which sarin was chromatographed initially, only Apiezon M and DC-LSX-3-0295 were considered to be useful. Further studies, in which account was taken of the column life, its separating efficiency and the possibility of applying temperature programming, have shown that QF-1, Carbowax 20M and EGSS-X polyester phase give good results[43,232].

The determination of sarin in water was carried out by extraction with chloroform, adsorption on Porapak Q, thermal desorption, and column chromatography[73]. This procedure and the application of FPD made it possible to determine the content of sarin in 1 ml of water at the picogram level. Tabun, soman and VX were also determined in water[77] and the suitability of FID and FPD was compared.

Sarin, soman, DFP, tabun and VX were determined in water by the headspace method[233]. Qualitative and quantitative analysis was carried out at the ppb level. However, difficulties were encountered when analysing tabun and VX.

The direct analysis of organophosphorus agents by GC doet nos usually present any major problems[234]. However, the verification of the presence of these compounds in the contaminated environment after a prolonged residence time may present difficulties. The physico-chemical effects that lead to the lowering of concentration and/or degradation of chemical warfare agents may affect significantly the results of analysis. In water samples, products of hydrolysis of organophosphorus compounds may be present. Griest and Martin[235] made a detailed study of their analysis[235]. Direct analysis of these substances is, in view of their polarity, very difficult. Therefore, they suggested that the hydroxy group be replaced with fluorine, which was achieved by treating the organophosphorus hydrolysis products successively with dicyclohexylcarbodiimide and hydrogen fluoride. A similar method was used in the analysis of phosphono- and phosphorothiolates after their reaction with silverfluoride[236].

Organophosphorus agents undergo transformations on prolonged storage. Analysis of tabun from chemical ammunition has shown that it contains five contaminants, and VX kept for 10–15 years in glass vessels was found to contain 23 impurities, including several that were not mentioned in earlier work[47,237].

As already mentioned, FPD is very useful for organophosphorus agents and ensures good detectability. It has been found that this detectability depends considerably on the molecular structure of the chemical warfare agents[238]. This relates either to different compounds or to one compound where the separation of its isomers is involved. The importance of the latter problem is connected with the fact that some isomers are much more deadly than others and their reactions with the live organism differ.

The simultaneous separation of isomers of sarin, soman and tabun was carried out by Degenhardt and co-workers[239,240]. They separated four stereoisomers of soman and enantiomers of sarin and tabun in a short capillary column filled with a mixed stationary phase containing a chiral component. Diastereoisomers of organophosphorus compounds may also be separated in conventional analytical columns with phases such as Triton X-305 or DC-550[241]. In this connection it has been shown for seventeen selected compounds that steric and electronic effects of the P–O–C and P–F bonds play a crucial role in the separation of organophosphorus esters.

In order to establish the interactions of the particular isomers of organophosphorus warfare agents with live organisms, it is important that these isomers be determined in biological samples[242,243]. It has been found that during the detoxication of soman in rat liver its rapid racemization takes place[244].

Several studies have dealt with the determination of soman and its isomers in blood[111–113,245–247]. For this purpose capillary columns with different stationary phases, including immobilized[111,112] and optically active types[113,246,247], were used. Separation into two[111,245] and four[113,246,247] isomers was achieved. The GC separation of four stereoisomers of soman is shown in Fig. 7.

In addition to soman, the content of sarin was determined in blood of dogs when studying the mechanism of blocking acetylcholinesterase and blood proteins by these warfare agents[111], DFP has also been determined in animal tissue[248]. Machata[249] described several chromatographic systems used for the analysis of this compound in the synthesis process.

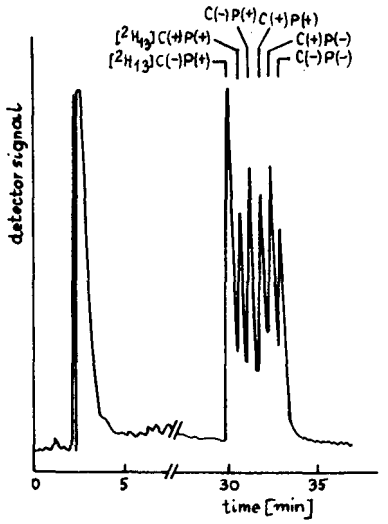



Fig. 7. Gas chromatogram of four stereoisomers [C(+)P(+)] of soman (50 pg) with internal standard C(+)P(+)[$^2H_{13}$]soman. Conditions: 50 m × 0.50 mm I.D. wide-bore capillary column; carrier gas, helium at a flow-rate of 2 ml/min; injection volume, 0.3 μl (direct injection); solvent, ethyl acetate; detection, AFID[113].

*5.3. Vesicant compounds*

Chromatographic methods were first used to analyse mustard gas in the mid-1960s. The application of FID and ECD methods made it possible to obtain satisfactory detectability of this chemical warfare agent. Today FPD with a 394-nm filter, specific for sulphur, is of particular importance as it allows the detection of mustard gas; other detection methods [coulometric Coulson detection (CCD) and Hall conductivity detection (HCD)] have also been applied to mustard gas[200].

Mustard gas often contains technological contaminants and decomposition products, *e.g.*, of hydrolysis[46,250]. The analysis has been described[250] of samples taken from chemical ammunition, soil and water which were collected from areas where the Iranian – Iraqi conflict took place. Most of the detected compounds were identified, some for the first time, and the relationship between their chemical structures and the retention parameters, were described.

A knowledge of the degradation mechanisms of vesicants makes it possible to determine the source and time of pollution by determining particular degradation products in the sample. By using GC – MS, the pollutants and products of decomposition of 2-chloroethylethyl sulphide, a product simulating mustard gas, were determined[251]. Samples stored for different periods were analysed in a capillary column. No products of oxidation or hydrolysis of the sulphide were detected. The main degradation product was 1,4-dithiane and a similar degradation mechanism to mustard gas was suggested.

The analysis of mustard gas and of the usually accompanying contaminants is conducted either in conventional analytical columns of length 0.6 – 3.0 m[40,41,46,200] or, for more complex samples (*e.g.*, biological), in capillary columns of length up to

15 m[109,250–253]. Stationary phases recommended for the analysis of mustard gas are SE-30[40], FFAP[41], QF-1[200] and SE-54[250].

In the analysis of mustard gas, tailing of peaks sometimes occurs owing to, among other things, the type of column material. The use of a PTFE column made it possible to avoid this undesirable effect[52].

Mustard gas in air is analysed after absorbing it in a non-volatile solvent[41,42]. Best solvent was diethyl succinate[42], which has more suitable properties for this purpose than hydrocarbon solvents[41].

GC may be used to assess the efficiency of protective clothing against mustard gas[40,254,255], as follows. The air containing mustard gas is passed first through the cloth from which the protective clothing is made and next through a washer with tetradecane[40]. The amount of mustard gas determined in the tetradecane allows reflects the protective efficiency of the clothing when account is taken of the concentration of mustard gas in air and the time of passage of the polluted air through the cloth.

Prior to 1982, no information was available on the use of GC for the detection of vesicants in biological materials. It was only after mustard gas was used in the Iranian – Iraqi conflict that various instrumental analytical methods, including GC, began to be used for the detection and identification of this agent in injured live organisms.

The identification of mustard gas in tissue and biological fluids (blood, serum, urine) immediately after intoxication is fairly easy[109,114,115,252,253]. Machata and Vycudilik determined mustard gas in urine of injured Iranian soldiers[109,252,253] using GC–MS with a quartz capillary column containing SE-54. The content of mustard gas determined in urine was 1–30 ppb. Heyndrickx *et al.*[114] determined mustard gas in biological samples and in soil at the picogram level using a capillary column with a non-polar phase and ECD.

It is much more difficult and sometimes even impossible to determine mustard gas in biological fluids after a certain time had elapsed after intoxication, because it undergoes complex metabolic processes. In this situation it is recommended that thiodiglycol, the main product of the hydrolysis of mustard gas is determined[118,119,253]. The analysis consists in converting this product by reaction with concentrated hydrochloric acid back to mustard gas, which is then isolated from the investigated biological material by the headspace method and subjected to GC–MS. This procedure has found, limited application, however, as it has been shown that with low thiodiglycol (55 ng/ml), it cannot be ascertained whether the mustard gas was produced from thiodiglycol or from some other substance of natural origin[118]. Recently a sensitive method for the determination of thiodiglycol in biological fluids after its conversion to bispentafluorobenzoate was reported[256]. By applying capillary GC–MS it was possible to detect thiodiglycol in amounts below 1 ng/ml in blood or urine samples.

Like mustard gas, lewisite may also be determined indirectly. Rózycki *et al.*[257] developed a method for determining lewisite in water consisting in the chromatographic determination of acetylene evolved in the reaction of *trans*-lewisite A with sodium hydroxide. In this way it was possible to determine lewisite in water at a $10^{-8}$% concentration.

*5.4. Irritants*

Irritants are used in form of vapour or aerosols dispersed in air. The most important are tear gases such as chloroacetophenone, *o*-chlorobenzylidenemalonodinitrile, chloropicrin, dibenzo[*b,f*]-1,4-oxazepine, camite and sternite – adamsite.

Because of the presence of halogens in the molecules of tear gases ECD is most commonly used for their detection[11,105,201,202,258]. This method allows the analysis of tear gases at the nanogram level, whereas the more convenient FID allows their analysis only at the microgram level[11,81,201,258,259]. Also detection methods, *e.g.*, NPD, TCD and argon-ionization ones, may also be used[201,258,260].

Martz *et al.*[225] compared mass spectrometric methods combined with GC. They used mass spectrometry with electron-impact ionization (EI), positive ion chemical ionization (PICI) and with negative ion chemical ionization (NICI). For CS gas analysis, NICI affords the best results.

GC–MS systems allows the rapid analysis of irritants with good sensitivity and reliable identification[63,105,261–264]. Wils and Hulst[63] determined CN, CS and CR by GC–MS at concentrations lowers than 1 ng/ml. To achieve such a high detectability they applied a special technique of injection into the capillary column of large samples (up to 250 $\mu$l). The analyte compounds dissolved in *n*-hexane or ethyl acetate were adsorbed in a column filled with Tenax GC. After thermal desorption, the compounds were trapped in a cold fused-silica capillary column (0.3 m x 0.5 mm I.D.) coated with CP-Sil 5 CB. Next the capillary was rapidly heated and the analyte compounds were desorbed and separated in a capillary column. The peaks obtained in the mass spectrum were identified. The presence of oxygen in the injector system resulted in the appearance of a peak of oxidized CS.

Sass *et al.*[201] determined CN, CS and CA and several of their characteristic contaminants, chiefly hydrolysis products. In order to prevent the decomposition of CA catalysed by the hot metal surface, some parts of the chromatograph were made of glass.

Jane and Wheals[202] developed a method for determining CN and CS in sprayers of tear gases. They tested many chromatographic columns for this purpose and it was found that a short analytical column made of stainless steel with Carbowax 20M as the stationary phase gave good results. The use ECD made it possible to determine CS and CN at the sub-nanogram level. The use of FID was difficult as the peaks of the lachrymators coincided with those of the solvents used in atomisers. Good results were obtained when combined detection methods, *e.g.*, FID–PND or FID–ECD, were used for the detection of CS[258].

Leadbeater *et al.*[105] analysed a CS metabolite in the blood of intoxicated cats and rats. They isolated the compound from the blood sample by its extraction with *n*-hexane or ethyl acetate.

Many studies have concentrated on the GC analysis of chloropicrin, as it is used as a component of plant protection agents (fumigants) and as a monitoring substance for testing the technical soundness of filtration equipment. In the analysis of chloropicrin, ECD[32,75,100,265–271] or MS[30,74,272,273] is recommended. Other types of detection such as HCD[270,271], CCD[274], TCD[275] or FID[276] have been used less frequently. Using GC, chloropicrin has been determined in water[30,32,74,75,268,269,273,277], grain and cereals[100,270,271,275,277,278], wine[265–267], food[75] and methyl bromide[279].

The determination of chloropicrin in water is conducted by two procedures.

The first consist in isolating chloropicrin together with other volatile halogenated organic contaminants by the headspace method and then subjecting them to GC–MS. The second procedure consists in extraction of the pollutants from water with an organic solvent (usually *n*-heptane), followed by chromatography with ECD.

For the determination of chloropicrin in water, either conventional analytical columns with squalane or silicones (DC-220, DS-550 or F-50) as stationary phases or capillary columns coated with phases such as DB-5, Durabond 1, OV-1 or SE-30 were used. These methods allow the determination of chloropicrin at the nanogram level in 1 $dm^3$ of water.

Daft[100,270,271,278], Berck[275,280] and Kanazawa[281] determined chloropicrin residues after the application of plant protection agents in cereals and fruit. For the chromatographic separation, various columns filled with single and mixed stationary phases were used, *e.g.*, OV-17, OV-101, SE-30, SP-1000, polyethylene glycol 6000 and OV-225–OV-17 (2:1). The main problem encountered in the analysis of fumigants was to find a solvent in which the analysed compounds would be stable and which would not be eluted in the same time as the compounds being analysed; isooctane proved to be the best[100]. When ECD or HCD was used, chloropicrin could be detected at the ppb level.

Chloropicrin in wine was determined after extraction with a non-polar solvent[265–267]. Quantitative determinations at the 10 $\mu g/dm^3$ level with the use of ECD were carried out by using trichloroethylene as the internal standard.

Sometimes it may be advantageous to determine chloropicrin indirectly after its conversion to ethylene chlorohydrin by reaction with ethylene oxide[285].

Analysis of adamsite by GC is difficult and the results are often irreproducible[259] and some workers claim that at present no effective and reliable method exists. The presence in the sample of diphenylamine (substrate for the synthesis of adamsite) makes the identification of the adamsite peak almost impossible. Despite this, some possibilities of analysing adamsite do exist. In the Helsinki report[10] the analysis of adamsite decomposition products on a capillary column coated with SE-52 or OV-1 was described. FID, ECD and ATD were mentioned as being useful, and with their use it was possible to detect adamsite at the $10^{-12}$ g level. The problems involved in the chromatographic analysis of organoarsenic compounds have been described in several papers[53,286–289].

### 5.5. *Fluoroacetic acid*

Fluoroacetic acid is a representative of toxic fluoroorganic compounds classified as potential chemical warfare agents. Its sodium salt is known as compound 1080. The high toxicity of this compound requires sensitive methods of analysis. The analysis of fluoroacetic acid by conventional chemical methods is not easy in view of the difficulties in splitting the strongly polarized bond between the fluorine and carbon atoms. GC is now the most common method[48,76,103,104,106,108,235,282–284,290–299]. For detection ECD[48,76,103,108,296], FID[104,282,292,293,298] and MS[106,284,295,297] have been used; the ion-selective fluoride electrode method[48,106,295], TCD[48,291,292] and PID[298] have been applied less frequently.

The first work on the analysis of fluoroacetic acid was published by Gershon and Renwick[292]. They separated lower 2-fluoroaliphatic acids on a short copper column. It was observed that fluoroacetic acid has a longer retention time than fluo-

ropropionic acid, which was the opposite of what would be expected from their structures and boiling points. This phenomenon was subsequently interpreted[293].

Stevens *et al.*[105] analysed fluoroacetic acid in a biological sample on a glass column filled with Porapak Q using FID. The determination of fluoroacetic acid was hindered as its peak coincides with those of other acids. They also attempted to apply GC–MS, but the results were unsatisfactory as considerable loses of the acid occurred owing to chemical reactions and/or adsorption on the metal surface of the metal tube connecting the GC column with the MS unit.

The conversion of fluoroacetic acid or its sodium salt into alkyl esters favours chromatographic analysis[48,104,106,282,290,293,295]. The production of fluoroacetic acid derivatives by reaction with *p*-bromophenacyl bromide[299], pentafluorobenzyl bromide[103,297], α-bromo-2,3,4,5,6-pentafluorotoluene[108] or N,N′-dicyclohexylcarbodiimide and 2,4-dichloroaniline[76] also proved advantageous.

Yu and Miller[104] analysed fluoroorganic acids in vegetable and animal tissues. It is assumed that the biosynthesis of toxic fluoroorganic acids due to addition of hydrogen fluoride to fragments of vegetable tissue is common for many tissues[96].

Sodium fluoroacetate is also of interest and has been determined in animal tissue[48,103,108,282,290,295–298], vegetable tissue[103,283,291], fungicides[249,290,298] and food[48]. The determination of compound 1080 in various materials was usually preceded by Soxhlet extraction with ethers, ketones or alcohols.

Casper *et al.*[297] achieved a high detectability (10 ppb) of sodium fluoroacetate by capillary GC–MS with a selective ion analyser.

Ozawa and Tsukioka[76] described a sensitive method for determining trace amounts of sodium fluoroacetate in water involving the use of ECD. This method consists in converting sodium fluoroacetate into the respective dichloroanilide derivative and chromatographic analysis of this derivative on a conventional glass column. The derivatives of other fatty acids did not interfere.

### *5.6. Hydrogen cyanide and cyanogen chloride*

Hydrogen cyanide and cyanogen chloride are very volatile and their determination in air is difficult in view of the rapid changes in their concentration. Therefore, to ascertain that they have been used and estimate their concentration in air, advantage is sometimes taken of the fact that in an aqueous medium these compounds yield cyanide ions, which are easy to detect[11,300].

The accuracy of the chromatographic analysis of hydrogen cyanide depends strongly on the way in which the sample was collected. The aspiration method involving the use of glass or metal containers is rejected because of adsorption of hydrogen cyanide on the walls of the containers. Instead, advantage is taken of the ready adsorption of hydrogen cyanide on porous materials, from which it can be extracted with, *e.g.*, *n*-hexane or desorbed thermally[33,60,92,301]. An interesting method of collecting samples was suggested by Kuessner[44], who presented two versions for collecting trace amounts of polar substances, and also hydrogen cyanide, at dry-ice temperature (−78°C). If the matrix of the sample was still gaseous at that temperature, the gas was passed through large washers filled with a suitable solvent. If, however, the matrix condensed at −78°C, then after absorption of the sample in a polar solvent the resulting solution was warmed slowly. In this way the matrix was isolated without any losses of the compounds to be analysed. The solutions of hydro-

gen cyanide (and other polar compounds) obtained by one of the above methods were analysed chromatographically.

Very good detectability of hydrogen cyanide has been reported[302,303]. In one method[302] hydrogen cyanide was detected at the level of 1 pg by thermionic nitrogen detection (TND) and in the other[303] 5 pg of hydrogen cyanide were detected with AFID.

Apart from conventional chromatographic detectors, for the analysis of hydrogen cyanide the procedure suggested by Cumming and Frost[304] can be applied. It is a general procedure for nitrogen-containing compounds, in which the components of the mixture, after leaving the chromatographic column, pass through a glass column filled with copper oxide or some other compound on which, at 700°C, these compounds are oxidized to nitrogen oxides which are subsequently detected by a chemiluminescence detector.

Hydrogen cyanide has been analysed not only in air but also in other media. Woolmington[57] determined hydrogen cyanide in a mixture of permanent gases and water vapour. The height of the hydrogen cyanide peak for a mixture containing water was slightly lower (by about 1%) than that for a sample free from water. This was explained by the selective adsorption of water by the strongly active sites of the support. Such an explanation seems highly probable in view of Berezkin's study of the gas–liquid–solid system[305]. The latter also provides an explanation of why long tailing of the hydrogen cyanide peak, characteristic of polar compounds occurring at low concentrations, was sometimes observed[306]. The addition of formic acid to the stream of carrier gas improves the detectability of hydrogen cyanide six-fold[307], owing to the decrease in the total adsorption activity of the support and the decrease in the association of hydrogen cyanide molecules.

In the analysis of hydrogen cyanide in mixtures of inorganic gases, medium-polarity liquid stationary phases, *e.g.*, glyceryl triacetate, dinonyl phthalate, poly (trifluorochloroethylene), and adsorbents such as Chromosorb 104 and Polisorb-1 are used in addition to polar liquid stationary phases and Porapaks[53,58,308–316]. The analysis is usually conducted on packed columns but sometimes capillary columns coated with the SE-52 or SE-54 phases are also used[11].

Hydrogen cyanide has frequently been analysed in combustion gases[302,306,317–320] and has been detected in the products of combustion of plastics[307,321–324] and wool[325].

As already mentioned, in aqueous solution hydrogen cyanide may yield cyanides. When such a solution is treated with acids stronger than hydrogen cyanide, the latter evolves from the solution and may easily be analysed on various chromatographic columns.

Another method of analysing hydrogen cyanide in water consists in isolating it by means of an inert gas. The latter, containing the hydrogen cyanide, is then passed through a bubbler in which hydrogen cyanide is absorbed in a suitable solvent[33,60,301,326]. This method gives good results if the concentration of hydrogen cyanide is above 5 ppb.

The analysis of hydrogen cyanide in biological samples has been described[116,327,328]. Hydrogen cyanide was isolated from blood by the headspace method and the gases evolved from blood heated to 60°C were passed through a PTFE column filled with Porapak QS[116].

GC has been used to determine hydrogen cyanide in plants, fruit and products of their processing[276,280,329–331]. With chlorinating agents, *e.g.*, chloramine T, hydrogen cyanide yields cyanogen chloride which, after dissolution in ethyl acetate, toluene or hexane, can easily be determined by GC[301,332,333].

A sensitive method of determining cyanides, consisting in conversion of cyanide ions into cyanogen chloride by reaction with chloramine T, has been applied to biological samples[40,334]. In the analysis of blood, urine and stomach contents, cyanogen chloride could be detected at the 30-pg level by using ECD. Special care was taken to minimize the losses of the volatile cyanogen chloride. The procedure requires relatively large samples and is laborious.

Brunnemann *et al.*[332] identified and determined hydrogen cyanide and cyanogen in tobacco smoke by chromatography with ECD after conversion to cyanogen chloride. The amount of hydrogen cyanide in one cigarette was found to exceed 50 μg. Brown *et al.*[335] also determined cyanogen chloride in the presence of cyanogen.

*5.7. Phosgene*

Phosgene is widely applied in industry as an intermediate for the synthesis of many compounds. In the atmosphere it is generated in the lower layers of the troposphere in smog containing various chlorine compounds. In addition, phosgene is generated in the course of the thermal or photochemical decomposition of halogen solvents. Phosgene is highly toxic, so monitoring its content in air is important, especially near workers and others who may be exposed to it. Pollution of air is also possible in cases of accidents or damage to chemical works.

The chromatographic analysis of phosgene is difficult in view of its high reactivity, which corrodes the chromatograph. In addition, at low concentrations it decomposes on contact with active surfaces. For these reasons the literature on the chromatographic analysis of phosgene was sparse for some time[292,336–342]. It was only after certain components of chromatographs were made of more inert materials (PTFE, nickel, niobium, tantalum or aluminium) that the number of studies on the analysis of phosgene by GC began to increase. A gas chromatograph resistant to aggressive gaseous compounds (HCl, $Cl_2$, $COCl_2$, $NO_2$), even in the presence of water, was described by Kuessner[343]. In this instrument all the surfaces that come into contact with the sample were made of glass or PTFE.

It has been shown[344–346] that the accuracy of phosgene analysis is affected by factors such as the flow-rate of the carrier gas and the size of the injected sample. Lillian and Singh[346] showed that samples of mass up to 0.1 ng did not affect the ionization efficiency of the electron-capture detector with respect to mass. In order to lower the detection limit they used a double system of electron-capture detectors in series and were able to detect phosgene at the femtogram ($10^{-15}$ g) level. Priestley *et al.*[339] found that the application of ECD allows phosgene to be determined at the 1–2 ppb level. The sensitivity of detection with respect to phosgene was comparable to that with respect to carbon tetrachloride (one of the best electron acceptors). In the analysis of phosgene other detectors have also been used, *e.g.*, the flame ionization detector, which allowed the detection of 0.3 μg of phosgene in 1 $dm^3$ of air[54], the coulometric flow-through detector[347,348], the modified Hall detector[349], mass spectrometers[30] and the detector in which use is made of a plasma discharge in argon with electrodeless excitation[350].

Phosgene has been determined in air alone[339,347,348,351–353] and in the presence of alkyl chloroformates[54], in various gas mixtures containing, *e.g.*, Ar and $CO_2$[336,354] or Ar, $N_2$, CO, $CO_2$, HCl and $Cl_2$[59,336,337,355], and also in the presence of volatile in organic chlorides[338,342,356]. Dahlberg and Kihlman[18] determined phosgene and acetyl chlorides generated in the decompostion of chloroorganic solvents and Reichert *et al.*[349] determined dichloroacetylene and its decomposition product phosgene. Many studies have been devoted to the analysis of inorganic and organic contaminants, including phosgene, in antimony, lead, titanium, tin, silicon and boron chlorides[292,338,341,357–361]. The relative retention times of phosgene and of some other chloroorganic compounds were given by Kiraly and Peter[362].

In the analysis of phosgene, chiefly liquid stationary phases were used in packed column. Capillary columns and adsorption chromatography were applied only in a few instances. Phosgene was analysed in the presence of argon and carbon dioxide, for instance, on a short silica gel column with temperature programming[354]. For more complex mixtures, systems of columns filled with liquid and solid stationary phases were used[59]. Some workers have recommended that, in view of the easy hydrolysis of phosgene, an initial adsorption column should be used to remove moisture[351–353].

Among liquid stationary phases, didecyl phthalate has been recommended for phosgene analysis[247,348]. For mixtures containing phosgene the selection of the stationary phases depends on the composition of the mixture. If acetyl chlorides were present in addition to phosgene, then the former were esterified and the resulting mixture was separated using silicone oil DC-200 or tridecyl phthalate as the stationary phase[18]. If alkylformates were present, they were converted into urea and carbamates, a normal column packed with neopentyl glycol succinate on Supelcoport or a capillary column coated with the DB-5 phase being used for their separation[54].

Phosgene is used for the derivatization of other compounds that are subsequently analysed by gas or liquid chromatography[197,363,364]. Gyllenhaal[365] applied derivatization for the indirect determinaton of phosgene and using a nitrogen detector he was able to determine 1 ng/ml levels.

Some examples of analysis of chemical warfare agents by GC are given in Table 4.

## 6. FINAL REMARKS

This review illustrates that chromatography is one of the most important, if not *the* most important, methods of analysis of chemical warfare agents. This conclusion reflects the well known fact that chromatographic methods are now most popular in organic analysis[366]. Chromatographic methods make it possible to analyse chemical warfare agents in complex mixtures, the detectability and sensitivity of determination being very good and the analysis times short. Various types of instruments may be used in automatic air control systems[314,367–369]. A simple instrument that combines a gas chromatograph with a mass spectrometer can be used even in field conditions[70]. This instrument may be utilized for the continuous analysis of chemical warfare agents in air and for their detection in water. Portable[370–373] and even pocket[374] chromatographs are also known.

The prospects for further progress in the analysis of chemical warfare agents by

TABLE 4

EXAMPLES OF ANALYSIS OF CHEMICAL WARFARE AGENTS BY GC

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| *Organophosphorus compounds* | | | | | |
| Nerve gases | 4.6 m x 0.32 mm I.D. fused-silica capillary columns; 0.15-μm OV-1, SE-52, SE-54, OV-1701; 0.20-μm Carbowax 20M, OV-351 | FID<br>ATD<br>ECD | pg | 30°C, 15°C/min to 200°C analysis of mixtures of agents based on 'retention spectrometry' | 13<br>229 |
| GB | 1.83 m x 2 mm I.D. Pyrex glass column; 3% EGSS-X on Gas-Chrom Q (100–120 mesh) | FPD | 0.3 ng | Nitrogen carrier gas; flow-rate, 20 ml/min; 89°C (2 min); 8°C/min to 200°C; determination in decontamination media | 43<br>232 |
| GA | 15 m x 0.32 mm I.D. capillary columns; 0.25-μm DB-1, DB-5, DB-1701 | FID<br>MS | | Helium carrier gas; linear flow-rate, 35 cm/s; 50°C (2 min), 10°C/min to 300°C; identification of 5 contaminants in munitions | 47 |
| GB | 1.83 m x 3 mm O.D. PTFE column; 5% QF-1–3% DC-220 on Gas-Chrom Q (60–80 mesh) | FPD | 67 pg/ml | Nitrogen carrier gas; flow-rate, 20 ml/min; 105°C; analysis in water | 73 |
| GA<br>GB<br>GD<br>VX | 1.83 m x 2 mm I.D. glass columns; 5% Carbowax 20M (GA) and 3% SP-2250 DB (VX); 1.83 m x 4 mm I.D. glass column; 10% SP-1000 (GB,GD) | FID<br>FPD | 0.1–0.5 ng<br>2–5 pg | Helium carrier gas; flow-rate, 15–30 ml/min; 130°C (GA), 170°C (GD,VX) and 150°C (3.5 min), 10°C/min to 160°C (GB); determination in water | 77 |
| GA<br>GB<br>GD<br>VX | 15 m x 0.32 mm I.D. fused-silica capillary columns; 0.25-μm DB-1, DB-5, DB-1701, DB-WAX | FID | | Helium carrier gas; linear flow-rate, 35 cm/s; 50°C (2 min), 10°C/min to 250°C (300°C) (5 min); determination of retention indices of 22 warfare agents and simulants | 91 |
| GB<br>GD | 15 m capillary column; SE-54-HP | MS | | 60°C (GB), 75°C (GD) and 60°C (4 min), 25°C/min to 250°C (GB,GD); analysis in dog blood | 111 |

| | | | | | |
|---|---|---|---|---|---|
| GD | 25 m x 0.22 mm I.D. fused-silica capillary column; CP Wax 57 | NPD | 40 pg/ml | Nitrogen carrier gas; flow-rate, 2 ml/min; 80°C (13 min); 40°C/min to 190°C (8 min); analysis in serum | 112 |
| GD | 50 m x 0.50 mm I.D. fused-silica capillary column; Chirasil Val (Type II); 48 m x 0.50 mm I.D. fused-silica capillary column; Chirasil Val (Type I); 14 m x 0.50 mm I.D. fused-silica capillary column; Carbowax 20M | AFID | 250 pg/ml | Helium carrier gas; flow-rate, 2 ml/min; 80°C; analysis of the four stereoisomers in rat blood | 113 |
| Nerve gases | 3 m x 3 mm I.D. borosilicate glass column; 10% FFAP on Chromosorb W HP (80–100 mesh) | AFID<br>ECD | 1 ppb | Argon–methane carrier gas; flow-rate, 30 ml/min; 120°C; analysis in water and soil | 233 |
| Nerve gases | 1.83 m x 3 mm O.D. stainless-steel column; 10% OV-61 on Chromosorb W (80–100 mesh) | FID<br>AFID | | Helium carrier gas; flow-rate, 25 ml/min; 183°C; analysis of organophosphorus compounds with hydroxyl groups in molecules | 235 |
| VX | 15 m x 0.32 mm I.D. fused-silica capillary columns; 0.25-$\mu$m DB-1, DB-5, DB-1701 | FID<br>MS | | Helium carrier gas; linear flow-rate, 35 cm/s; 50°C (2 min), 10°C/min to 280°C (10 min); detection of 23 contaminants in VX | 237 |
| GA<br>GB<br>GD<br>VX | 1.83 m x 2 mm I.D. Pyrex glass columns; 10% QF-1 on Gas-Chrom Q (80–100 mesh); SE-30 on Gas-Chrom P (80–100 mesh) | FID<br>FPD<br>NPD | | Nitrogen carrier gas; flow-rate, 75 ml/min; or helium carrier gas; flow-rate, 40 ml/min (NPD); 60°C, 8°C/min to 200°C | 238 |
| GA<br>GB | 2 m x 0.44 mm I.D. glass capillary column; bis[(1*R*)-3-(heptafluorobutyryl)camphorate]nickel(II) in OV-101 | NPD<br>MS | pg | Helium carrier gas; linear flow-rate, 50 cm/s; 120°C; separation of stereoisomers; analysis of GA in biological fluids of animals | 239 |
| GB | 2.7 m x 3 mm I.D. glass column; 25% DC-550 on Chromosorb W AW (80–100 mesh); or 3 m x 4 mm I.D. glass column; 25% Triton X-305 on Chromosorb W AW (80–100 mesh) | FID | | Nitrogen carrier gas; flow-rate, 20 ml/min; 110°C | 241 |
| GD | 1.5 m glass column; 10% SP-1200–1% $H_3PO_4$ on Chromosorb W AW DMCS (80–100 mesh) | MS | | 95°C (1 min), 8°C/min to 130°C; analysis of stereoisomers in rat liver | 244 |

*(Continued on p. 388)*

TABLE 4 (*continued*)

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| GD | 20 m x 0.32 mm I.D. glass capillary column; SP 1000 WCOT | MS | 5 ng/ml<br>5 ng/g | Helium carrier gas; flow-rate, 2 ml/min; determination in nerve tissue and blood of mice | 245 |
| GD | 25 m x 0.3 mm I.D. capillary column; Chirasil-Val; 30 m x 0.3 mm I.D. capillary column; Carbowax 20 M–Chirasil-Val | FID | | 80°C; separation 4 stereoisomers in rat blood, in liver homogenates of rats and on passage through excised guinea pig skin | 246 |
| GD | 2 m x 0.44 mm I.D. glass capillary column; bis[(1*R*)-3-(heptafluorobutyryl)camphorate]nickel(II) in OV-101 | AFID | 30 pg per 3 ml | Separation of 4 stereoisomers in blood | 247 |
| DFP | 15% XF-1150 on Gas-Chrom Q | FPD | < 1 ng | Analysis of residues in pig tissues | 248 |
| GB | 1.83 m x 3 mm O.D. PTFE column; 3% DC-200–5% QF-1 on Gas-Chrom Q (60–80 mesh) | FPD | 0.1 $\mu g/dm^3$ | Nitrogen carrier gas; flow-rate, 30 ml/min; 120–150°C; automated analysis of air | 367 |
| *Vesicant compounds* | | | | | |
| HD | 4.6 mm x 0.32 mm I.D. fused-silica capillary columns; 0.15 $\mu$m, OV-1, SE-52, SE-54, OV-1701; 0.20-$\mu$m, Carbowax 20M, OV-351 | FID<br>ATD<br>ECD | ng | 30°C, 15°C/min to 200°C; analysis of mixtures of agents based on 'retention spectrometry' | 13<br>229 |
| HD | 3 m x 3 mm O.D. stainless-steel column; 10% SE-30 on Diatoport S (60–80 mesh) | FID | 5 ng | Helium carrier gas; flow-rate, 60 ml/min; 125°C | 40 |
| HD | 0.61 m x 3 mm I.D. stainless steel column; 2% FFAP on Chromosorb W AW DMCS (60–80 mesh) | ECD | 0.2 ng | Argon–methane (95:5) carrier gas; flow-rate, 23 ml/min; 150°C; air analysis | 41 |
| HD | 1.83 m x 3 mm O.D. stainless-steel column; 4% FFAP on Chromosorb W AW DMCS (60–80 mesh) | FPD | 0.2 ng | Nitrogen carrier gas; flow-rate, 55 ml/min; 155°C; air analysis | 42 |

| | | | | | |
|---|---|---|---|---|---|
| HD | 1.5 m x 2 mm I.D. glass column, 3% cyclohexanedimethanol succinate on Gas-Chrom Q (100–120 mesh) | FID | μg | Helium carrier gas; flow-rate 35–40 ml/min; 120°C or 110°C, 28°C/min to 230°C; separation of HD, halfmustard and thiodiglycol | 46 |
| HD | 1.83 m x 3 mm O.D. PTFE column 2% SE-30 and 5% Carbowax 4000 on Chromosorb 750 (60–80 mesh) | FPD | ng | Nitrogen carrier gas; flow-rate, 25 ml/min; 140°C; air analysis | 52 |
| HD | 15 m x 0.32 mm I.D. fused-silica capillary columns; 0.25-μm DB-1, DB-5, DB-1701, DB-WAX | FID | | Helium carrier gas; linear flow-rate 35 cm/s; 50°C (2 min), 10°C/min to 250°C (300°C) (5 min); analysis in soil; determination of retention indices of 22 warfare agents and simulants | 91 |
| HD | 25 m x 0.32 mm I.D. fused-silica capillary column; polydiethylsiloxane (CP tm Sil S) | ECD | pg | Argon–methane (90:10) carrier gas; 55°C (5 min), 5°C/min to 120°C, 10°C/min to 300°C (10 min); analysis in biological samples and soil | 114 |
| HD<br>HN-3 | 1.83 m x 6 mm O.D. Pyrex glass column; 10% QF-1 on Gas-Chrom Q (60–80 mesh) or 3% QF-1 on Gas-Chrom Q (100–120 mesh) | FID<br>ECD<br>FPD-S,P<br>HCD | 40 ng<br>0.2 ng<br>0.7–2 ng<br>5 ng | Helium carrier; flow-rate, 40 ml/min; or argon–methane (90:10) (ECD); flow-rate, 90 ml/min; 100°C or 60°C, 8°C/min to 230°C | 200 |
| HD | 15 m x 0.32 mm I.D. fused-silica capillary columns; 0.25-μm DB-1, DB-5, DB-1701 | FID | | Helium carrier gas; linear flow-rate, 35 cm/s; 50°C (2 min), 10°C/min to 300°C (5 min); determination of retention indices for 37 sulphur vesicant and vesicant-related compounds | 251 |
| HD | 15 m x 0.32 mm I.D. fused-silica capillary column; SE-54 | MS | 10 ng/μl | Helium carrier gas; flow-rate, 6 ml/min; 50°C, 20°C/min to 280°C; urine analysis | 252 |
| L | 2.1 m x 4 mm I.D. glass column; silica gel (30–75 mesh) | FID | $10^{-7}$ g/dm$^3$ | Nitrogen carrier gas; flow-rate, 40 ml/min; 180°C; analysis in water | 257 |
| HD | 1.83 m x 3 mm O.D. PTFE column, 3% DC-200–5% QF-1 on Gas-Chrom Q (60–80 mesh) | FPD | 3 μg/dm$^3$ | Nitrogen carrier gas; flow-rate, 30 ml/min; 120–150°C; automated analysis of air | 367 |

*(Continued on p. 340)*

TABLE 4 (*continued*)

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| *Irritant compounds* | | | | | |
| CN<br>CR<br>CS | 4.6 m x 0.32 mm I.D. fused-silica capillary columns; 0.15 μm, OV-1, SE-52, SE-54, OV-1701; 0.20 μm, Carbowax 20M, OV-351 | FID<br>ATD<br>ECD | ng | 30°C, 15°C/min to 200°C; analysis of mixtures of agents based on 'retention spectrometry' | 13<br>229 |
| CN<br>CR<br>CS | 50 m x 0.3 mm I.D. fused-silica capillary column; Chrompack (CP Sil 8 CB) | MS | < 5 ng | Nitrogen carrier gas; flow-rate, 30 ml/min; 80°C, 10°C/min to 240°C | 63 |
| CN<br>CR<br>CS | 15 m x 0.32 mm I.D. fused-silica capillary columns; 0.25-μm DB-1, DB-5, DB-1701, DB-WAX | FID | | Helium carrier gas; linear flow-rate, 35 cm/s; 50°C (2 min), 10°C/min to 250°C (300°C) (5 min); determination of retention indices of 22 warfare agents and simulants | 91 |
| CS | 1.5 m x 3.1 mm I.D. Pyrex glass columns; 10% polyethylene glycol adipate or 10% Apiezon L on Celite (100–200 mesh); 0.4 m x 2.2 mm I.D. stainless-steel column; 5% phenyldiethanolamine succinate on Chromosorb G (85–100 mesh) | ECD<br>MS | | Nitrogen carrier gas; flow-rate, 60 ml/min; 185°C; and helium carrier gas (MS); flow-rate, 15 ml/min; 170°C; analysis in cat and rat blood | 105 |
| CA<br>CN<br>CS | 1.7 m x 6 mm O.D. borosilicate glass column; 10% QF-1 on Gas-Chrom Q (60–80 mesh) | TCD<br>FID<br>ECD | mg<br>μg<br>4 ng (CA)<br>0.2 ng (CN)<br>0.1 ng (CS) | Helium carrier gas; flow-rate, 90 ml/min; 130°C (CA,CN), 150°C (CS) and 65°C, 6°C/min to 200°C; detection and determination in gases and liquids | 201 |
| CN<br>CS | 0.9 m x 2.2 mm I.D. stainless-steel column; 2% Carbowax 20M on Chromosorb G (80–100 mesh) | ECD<br>FID | > 0.2 ng<br>2–5 ng | Nitrogen carrier gas; flow-rate, 30 ml/min; 60 and 180°C; analysis in tear gas aerosols | 202 |

| | | | | | |
|---|---|---|---|---|---|
| CS | 1.5 m x 4 mm I.D. glass column; 3% SE-30 on Chromosorb G-HP (80–100 mesh) (I); or 4 m x 3 mm I.D. glass column; 3% OV-1 on Chromosorb W HP (80–100 mesh)(II) | FID–ECD<br>FID–NPD | | Nitrogen carrier gas; flow-rate, 60 ml/min (I); and helium carrier gas; flow-rate, 50 ml/min (II); determination of retention indices for 296 substances | 258 |
| CN<br>CS<br>DM | 1.83 m x 3 mm I.D. Pyrex glass column; 3% OV-17 on Varaport 30 (100–120 mesh) | FID | μg | Nitrogen carrier gas; flow-rate, 25 ml/min; 55°C, 12°C/min to 210°C; analysis in mixtures; with other irritants and their impurities | 259 |
| CN<br>CS | 1.83 m x 3 mm I.D. glass column; 3% OV-1 on Gas-Chrom Q (60–80 mesh) | AID | μg | Argon carrier gas; flow-rate, 50 ml/ml; 145°C; analysis in blood and tissue | 260 |
| CN | 1 m column; 5% OV-17 | MS | ng | 25°C (22 min), 8°C/min to 250°C | 262 |
| PS<br>CG | 25 m x 0.25 mm I.D. silica gel capillary column; SE-30–OV-1 (1:1) | MS | | Helium carrier gas; 0–180°C (200°C); determination of volatile organic compounds in decontaminated water | 30 |
| PS | 25 m x 0.2 mm I.D. fused-silica capillary column; 0.3-μm OV-1 (I); or 3 m x 2 mm I.D. glass column; 10% Squalane on Chromosorb W AW (80–100 mesh)(II) | ECD | | Helium carrier gas; 35°C (1 min), 20°C/min to 70°C (1 min), 3°C/min to 225°C (I); or argon–methane carrier gas; flow-rate, 25 ml/min; 67°C (II); analysis in drinking water | 32 |
| PS | 30 m x 0.32 mm I.D. fused-silica fused capillary column; 1.0 μm DB-5 | MS | | Helium carrier gas; −50°C, 30°C/min to 50°C, 5°C/min to 240°C; identification in groundwater | 74 |
| PS | 60 m fused-silica capillary column; Durabond 1 | ECD | 10 pg | Nitrogen carrier gas; 40°C (35 min), 5°C/min to 160°C; analysis of 16 chloroorganic compounds in water, waste water and food | 75 |
| PS | 1.83 m x 4 mm I.D. glass columns; 20% OV-225–20% OV-17 (2:1), 20% OV-101 and 20% OV-17 on Chromosorb W HP (80–100 mesh) | ECD | ppm | 75–85°C; analysis of 7 fumigants in food | 100 |
| PS | 10% SE-30 on Chromosorb W AW (80–100 mesh) | ECD | | Analysis in wine | 266 |

*(Continued on p. 342)*

TABLE 4 (*continued*)

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| PS | QF-1, QV-17 on Chromosorb W (80–100 mesh) | ECD | < 0.01 mg/dm$^3$ | Analysis in wine and high alcoholic beverages | 267 |
| PS | Glass columns with squalane, silicone DC-200, silicone Versilube F-50 | ECD | 0.06 μg/dm$^3$ | Analysis in drinking water with other halogenated compounds | 269 |
| PS | 1.8 m x 4 mm I.D. or 3.6 m x 4 mm I.D. glass columns; 20% OV-101, 10% SP-1000, 20% OV-17 and 20% OV-225–20% OV-17 (2:1) on Chromosorb W (80–100 mesh) | ECD<br>HCD | 0.2 ng | 80–90°C; determination of 10 fumigant residues in grain and grain-based products | 270 |
| PS<br>AC | 1.83 m x 6 mm O.D. stainless-steel column; 10% SE-30 on Diatoport S (60–80 mesh) | TCD | | 50°C, 15°C/min to 180°C; separation of 34 fumigants | 275 |
| PS | 50 m x 0.2 mm I.D. fused-silica capillary column; SP-1000 WCOT | FID | | Nitrogen carrier gas; linear flow-rate, 10 cm/s; 20°C (5 min), 2°C/min to 250°C; determination of retention indices for 221 chloroorganic compounds | 275 |
| | | *Industrial compounds* | | | |
| $FCH_2COOH$ | 2 m x 4 mm I.D. glass columns; 10% polyethylene glycol 6000 (I) and 10% Reoplex 400 (II) on Chromosorb W AW DMCS (60–80 mesh) | FID | | Helium carrier gas; 65°C, 3°C/min to 215°C (I) and 50°C (60°C), 43°C/min to 200°C (II); analysis in plant tissue and organs of animals | 104 |
| $FCH_2COOH$ | 1.5 m x 4 mm I.D. glass column; Porapak Q | MS<br>FID | 0.1 μg/g | Helium carrier gas; flow-rate, 30 ml/min; 200°C; determination in animal tissues | 106 |
| $FCH_2COOH$ | 0.4 m x 6 mm I.D. column; 10% Reoplex 400 on Chromosorb W (60–80 mesh) | TCD | | Analysis of 10 carboxylic acids in plant samples | 288 |

| | | | | | |
|---|---|---|---|---|---|
| $FCH_2COOH$ | 1 m x 6 mm O.D. copper column; 15% Tween 80–$H_3PO_4$ (9:1) on Chromosorb W (30–60 mesh) (I); 2.2 m x 3 mm O.D. copper column; 10% Tween 80–$H_3PO_4$ (9:1) on firebrick (100–120 mesh)(II) | TCD<br>FID | | Helium carrier gas; flow-rate, 90 ml/min; 132°C (I); nitrogen carrier gas; flow-rate, 30 ml/min; 156°C (II); separation and identification of $C_2$–$C_6$ fluorinated fatty acids | 292 |
| $FCH_2COOH$ | 1.5 m x 3 mm O.D. stainless-steel column; 5% DEGS on Chromosorb W (80–100 mesh) | FID | | Nitrogen carrier gas; flow-rate, 25 ml/min; 85 or 100°C, 5°C/min to 200°C; analysis of mixtures of methyl esters of 2-fluoro-fatty acids up to $C_{18}$ | 293 |
| $FCH_2COONa$ | 1.83 m x 3 mm I.D. or 1.83 m x 6 mm I.D. glass columns; Resoflex; 7.6 m x 3 mm I.D. or 7.6 m x 6 mm I.D. glass column; Resoflex | TCD<br>ECD | <br>0.1 μg | Nitrogen carrier gas; flow-rate, 50 ml/min; 75°C; analysis in biological tissue, food and baits | 48 |
| $FCH_2COONa$ | 2.1 m x 3 mm I.D. glass columns; Apiezon L–$H_3PO_4$ (5 + 2%) and DEGS–$H_3PO_4$ (5 + 1%) on Chromosorb W (60–80 mesh) | ECD | 0.03 ng | Nitrogen carrier gas; flow-rate, 20 ml/min; 175°C; determination in water | 76 |
| $FCH_2COONa$ | 1.83 m × 2 mm I.D. Glass column; 3% DC-200 or 3% DC-200 and 5% QF-1 (1:1) on Chromosorb W (100–120 mesh) | ECD | 0.2 ppm | Argon–methane (90:10) carrier gas; flow-rate, 20 or 40 ml/min; 105°C; determination in grain samples and animal tissues | 103 |
| $FCH_2COONa$ | 1.83 m x 3 mm I.D. or 1.83 m x 6 mm I.D. glass columns; Resoflex | MS | 0.1 μg | Nitrogen carrier gas; flow-rate, 50 ml/min; 75°C; determination in biological samples | 295 |
| $FCH_2COONa$ | 0.9 m x 3 mm I.D. aluminium column; Chromosorb 102 (100–120 mesh) | FID | 0.1–0.2 ppm | Nitrogen carrier gas; flow-rate, 30–35 ml/min; 155 or 180°C; determination in plant tissue | 282 |
| AC | 1.0–5 m columns; Porapak T or Q | TCD<br>FID | 1 ppb<br>1 ppm | Hydrogen carrier gas; flow-rate, 50 ml/min; 120°C (130°C) or 120°C (2 min), 5°C/min to 145°C (5 min), 25°C/min to 200°C (11 min); analysis in gaseous mixture | 44 |
| AC | 2.1 m x 8 mm O.D. stainless-steel column; 20% polyethylene glycol 1500 on Chromosorb or Celite (30–60 mesh); and 1.0 m x 6 mm I.D. glass column; molecular sieve 5A | TCD | | Analysis in gaseous mixture | 57 |

*(Continued on p. 344)*

TABLE 4 (*continued*)

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| AC | 2.4 m x 5 mm I.D. copper column; 25% glyceryl triacetate on Chromosorb P (30–60 mesh) (I); and 2.7 m x 5 mm I.D. copper column; molecular sieve 5A or 13X (II) | TCD | | 75°C (I) and 20°C (II); determination AC and cyanogen in presence of $CO_2$, $O_2$, $N_2$, $CH_4$ and CO | 58 |
| AC | 5.5 m x 6 mm I.D. stainless-steel column; 20% dinonyl phthalate on Chromosorb W | TCD | 25 $\mu g/dm^3$ | Helium carrier gas; flow-rate, 135 ml/min; 44°C; analysis in water | 60 |
| AC | 25 m x 0.22 mm I.D. fused-silica capillary column; CP-SIL 5 | MS | | Helium carrier gas; 0°C, 3°C/min to 275°C; analysis in soil by pyrolysis gas chromatography | 92 |
| AC | 1.83 m x 3 mm O.D. PTFE column; Porapak QS | ATD | $5 \cdot 10^{-8}$ g/ml | Helium carrier gas; flow-rate, 30 ml/min; 80°C; analysis in blood | 116 |
| AC | 1.83 m x 6 mm O.D. stainless-steel column; 10% SE-30 on Diatoport S (60–80 mesh) | TCD | | 50°C, 15°C/min to 180°C; separation of 34 fumigants | 275 |
| AC | 0.9 m x 2 mm I.D. glass column; Chromosorb 101 (80–100 mesh) | TID-N | 1 pg | Air analysis | 302 |
| AC | 3.6 m x 3 mm O.D. stainless-steel column; 5% Carbowax 400 on Teflon 6 | FID | $<10$ ppm | Helium carrier gas; flow-rate, 21 ml/min; 60°C; determination in hydrocarbon combustion effluents | 306 |
| AC | 2 m x 3 mm I.D. stainless-steel column; Silochrom S-80 with 4–8% $H_3PO_4$ | FID | | Argon carrier gas; flow-rate, 33 ml/min; 100°C; determination of pyrolysis products of poly-urethane foams | 307 |
| AC | 6 m x 6 mm I.D. glass column; Daifuloil No. 1, 3 and 50 [poly(trifluorochloroethylene)] on Polifulon powder (30–60 mesh) | TCD | | Ambient temperature; analysis of reactive inorganic gases and vapours | 308 |

| | | | | | |
|---|---|---|---|---|---|
| AC | 3 m x 3 mm I.D. column; Polisorb 1 | TCD | | 45°C; analysis of hydrogen sulphide gas | 312 |
| AC | 2 Columns, 5–7% cyanoethylated pentaerythritol and 2–7% diglycerol on Polisorb 1 | TCD | | 95°C; analysis of products of oxidative ammonolysis of propylene | 313 |
| AC | 2.8 m x 4 mm I.D. stainless-steel column; 20% dinonyl phthalate on Dinokhrom N | TCD | | 54°C; determination with $(CN)_2$ | 315 |
| AC | 3 m Column; Porapak Q (50–100 mesh); 2.5 m column, 15% polypropylene glycol on Chromosorb AW DMCS (60–70 mesh) | | | 35°C; analysis of exhaust gases | 317 |
| AC | 3 m x 4 mm I.D. column; Porapak Q | FID | < 0.1 ppm | 30°C, 2°C/min to 50°C, 5°C/min to 200°C; determination in exhaust gases | 318 |
| AC | 2 m x 1 mm I.D. glass column; 7% Carbowax 20M on Chromosorb W (80–100 mehs); and 4 m x 4 mm I.D. glass column, 7% triacetin on Chromosorb W (80–100 mesh) (I); 2 m x 2 mm I.D. stainless-steel column, 5% dimethylsulpholane on Inerton AW (90–120 mesh) (II) | FID<br>TCD | $7 \cdot 10^{-10}$ g<br>$3.5 \cdot 10^{-7}$ g | Helium carrier gas; flow-rate, 30 ml/min; 40°C (I) and 20°C (II); quantitative analysis of pyrolysis products formed during thermal degradation of polymers | 324 |
| AC | 2 m x 3 mm I.D. column; Porapak Q | | | Determination in unsweetened bean jam | 330 |
| AC | 1.83 m x 6 mm O.D. stainless-steel column; 7% Halcomid M-18 on Anakrom AB5 (90–110 mesh) | ECD | $12.5 \cdot 10^{-12}$ g | Argon–methane (95:5) carrier gas; flow-rate, 40 ml/min; 55°C; determination of cyanide in biological samples | 334 |
| CK | 1.5 m x 3 mm I.D. column; Porapak QS | FID<br>TCD | $2 \cdot 10^{-11}$ mol<br>$4 \cdot 10^{-11}$ mol | Helium carrier gas; flow-rate, 34.5 ml/min; 90°C; comparative study of the response two detectors to CK and $(CN)_2$ | 335 |
| CG | 2 m x 3 mm I.D. stainless-steel column; 20% silicone oil DC-200 on Chromosorb W | ECD | < 1 ppb | Argon–methane (95:5) carrier gas; flow-rate, 30 ml/min; 25°C; analysis of mixture of acetyl chlorides in air | 18 |
| CG | 25 m x 0.25 mm I.D. silica gel capillary column; SE-30 and OV-1 (1:1) | MS | | Helium carrier gas; 0–180°C (200°C); determination of volatile organic compounds in decontaminated water | 30 |

*(Continued on p. 346)*

TABLE 4 (*continued*)

| *Chemical warfare agent* | *Column characteristics* | *Detection* | *Detection limit* | *Conditions of analysis and remarks* | *Ref.* |
|---|---|---|---|---|---|
| CG | 3 m x 3 mm O.D. nickel column; 10% neopentyl glycol succinate on Supelcoport (100–120 mesh); or 30 m x 0.32 mm I.D. fused-silica capillary column; DB-5 | FID | 0.7 μg | 190°C or 150°C (16 min), 16°C/min to 190°C; simultaneous determination of CG and chloroformates in air | 54 |
| CG | 1 m x 6 mm I.D. stainless-steel column; 28.3% Apiezon N on Sterchamol | TCD | | Hydrogen carrier gas; 100°C; analysis of impurities in $SiCl_4$, $SnCl_4$ and $TiCl_4$ | 338, 341 |
| CG | 2 m x 4.7 mm I.D. aluminium column; 30% Flexol 10-10 (didecyl phthalate) on GC-22 Super Suport (100–120 mesh) | ECD | 2 ng | Nitrogen carrier gas; flow-rate, 50 ml/min; 50°C; automated air analysis | 339 |
| CG | 3 m x 6 mm O.D. aluminium column; 30% didecyl phtalate on Chromosorb P (100–120 mesh) | 2 ECD | $10^{-15}$ g | Nitrogen carrier gas; flow-rate, 37 ml/min; 23°C; determination in air | 347 |
| CG | 5.5 m x 6 mm I.D. aluminium column; 30% silicone oil DC-200 on Chromosorb P (100–120 mesh) | 2 ECD | 0.28 ppm | Nitrogen carrier gas; flow-rate, 60 ml/min; 23°C; analysis of 8 halogenated hydrocarbons in urban air | 348 |
| CG | 1.83 m x 2 mm I.D. column; 30% Squalane on Chromosorb W AW (100–120 mesh) | ECD | ppb–ppm | Helium carrier gas; flow-rate, 30 ml/min; 80°C; detection of dichloroacetylene and its decomposition product CG | 349 |
| CG | 2.5 m x 1.5 mm I.D. aluminium column; 30% diisodecyl phthalate on Aeropak (80–100 mesh) | ECD | 0.02 ppm | Nitrogen carrier gas; flow-rate, 30 ml/min; 50°C; determination of CG and dichloroacetylene in air | 351 |

| | | | | | |
|---|---|---|---|---|---|
| CG | 3 m x 3 mm I.D. PTFE column; Chromosil 310 | ECD | 1 ppb | Argon–methane (95:5) carrier gas; flow-rate, 15 ml/min; 30°C; air analysis | 352 |
| CG | 0.15 m x 3 mm O.D. column; silica gel A-40 (30–60 mesh) | TCD | | Helium carrier gas; flow-rate, 20.5 ml/min; 30°C (3 min)–150°C; analysis of CG, $CO_2$ and Ar mixture | 354 |
| CG | 9 m x 4.2 mm I.D. PTFE column; 10% Arochlor 1232 on Chromosorb T (40–60 mesh) | Mass detector | | Helium carrier gas; flow-rate, 200 ml/min; 32°C; analysis in CO, $CO_2$, HCl and $Cl_2$ mixture | 355 |
| CG | Glass column; 10–15% polymethylsiloxane PMS-20, PMS-100, PMS-200 on Spherochrome 1 | TCD<br>TID | | Nitrogen carrier gas; analysis of inorganic compounds in $SiCl_4$ and $GaCl_3$ | 357 |
| CG | 0.4 m x 4 mm I.D. column; 15% polyethylene glycol 6000 or polymethylsiloxane PMS-100 on Hezasorb | FID<br>TCD<br>MS | | Nitrogen carrier gas; flow-rate, 26 ml/min; 140°C; analysis of purity of $PbCl_4$ and $SbCl_4$ | 358 |
| CG | 4 m x 4.5 mm I.D. glass column; 16% dinonyl phthalate or 16% silicone elastomer E-301 on Chromaton N AW HMDS | FID<br>TCD | | Nitrogen carrier gas; flow-rate, 45 ml/min; 50°C; determination of impurities in $SiCl_4$ | 359 |
| CG | 0.6 m x 4 mm I.D. column; 20% diphenylamine on Spherochrome 1 | MS | | Helium carrier gas; 140°C; identification of impurities in $PbCl_4$ | 361 |
| CG | 1.2 m x 2 mm I.D. glass column; 3% Hi-EFF-BBP on Gas-Chrom Q (100–120 mesh); and 0.6 m x 2 mm I.D. glass column; 3% Carbowax 20M on Gas-Chrom Q (100–120 mesh) | NPD | 1 ng/ml | Nitrogen carrier gas; flow-rate, 30 ml/min; 200 or 240°C; determination after cyclization with a 2-hydroxyamine | 365 |

chromatographic methods are very good, owing to the progress in the collection and preparation of samples for chromatographic analysis[38,375–380] and the development of particular chromatographic methods[381,382]. The latter relates especially to GC[383–388] and TLC[389,390]. The recent rapid development of supercritical fluid chromatography (SFC) also deserves attention. It seems that SFC, which has not so far been applied in the analysis of chemical warfare agents, may be particularly useful for the purpose[391–395].

Since the completion of the literature survey, a number of papers relevant to the chromatographic determination of chemical warfare agents have been published[396–413]. Among the agents studied were PS[396,398,404,406], HD[397,399,401], GA[400,405], GB[400,403,405], GD[400,401,405], VX[400,403,405,412], BZ[402], PF-3[405] and sodium monofluoroacetate[410].

## 7. SUMMARY

The usefulness and applications of the particular types of chromatography in the analysis of chemical warfare agents have been reviewed. A major problem in the chromatographic analysis of chemical warfare agents is the collection and preparation of the samples. The importance of this problem differs for the various types of chromatography. Significant differences occur in the way in which samples are collected from air, water, soil, vegetables or animal organisms.

The analyses are characterized by the main groups of chemical warfare agents, *e.g.*, organophosphorus, vesicants, irritants, etc. Account has been taken of the relationships between their properties and the possibilities of their chromatographic analysis. The advantages and disadvantages of particular types of chromatography in the analysis of the particular groups and individual agents have been considered. The detectability of particular chemical warfare agents has been assessed, together with the separating efficiency for their mixtures. Examples of applications of chromatographic systems and conditions of chromatographing are summarized in tables.

It is concluded that chromatography is a very useful tool in the analysis of chemical warfare agents; GC and TLC have the most advantageous properties, HPLC being slightly inferior.

## REFERENCES

1 S. Budiansky, *Nature (London)*, 308 (1984) 483.
2 G. Baumann, *Zivilverteidigung*, 17 (1986) 5.
3 *Report of the Specialists Appointed by the Secretary-General to Investigate Allegations by the Islamic Republic of Iran Concerning the Use of Chemical Weapons, Report S/16433*, United nations, New York, 26 March 1984.
4 R. Stohr, T. Stock and K. Muller, *Z. Chem.*, 27 (1987) 117.
5 K. Lohs, T. Stock and V. Klass, *Z. Chem.*, 27 (1987) 349.
6 *Chemical and Instrumental Verification of Organophosphorus Warfare Agents*, Ministry for Foreign Affairs of Finland, Helsinki, 1977.
7 *Identification of Organophosphorus Warfare Agents. An Approach for the Standardization of Techniques and Reference Data*, Ministry for Foreign Affairs of Finland, Helsinki, 1979.
8 *Identification of Degradation Products of Organophosphorus Warfare Agents*, Ministry for Foreign Affairs of Finland, Helsinki, 1980.
9 *An Approach to the Environmental Monitoring of Nerve Agents*, Ministry for Foreign Affairs of Finland, Helsinki, 1981.

10 *Identification of Non-phosphorus Warfare Agents*, Ministry for Foreign Affairs of Finland, Helsinki, 1982.
11 *Identification of Precursors of Warfare Agents, Degradation Products of Non-phosphorus Agents and Some Potential Agents*, Ministry for Foreign Affairs of Finland, Helsinki, 1983.
12 *Scientific Methods for the Verification of Chemical Disarmament*, Ministry for Foreign Affairs of Finland, Helsinki, 1984.
13 *Air Monitoring as a Means for the Verification of Chemical Disarmament, Part 1, Development and Evaluation of Basic Techniques*, Ministry for Foreign Affairs of Finland, Helsinki, 1985.
14 *Air Monitoring as a Means for the Verification of Chemical Disarmament, Part 2, Field Tests*, Ministry for Foreign Affairs of Finland, Helsinki, 1986.
15 *Trichothecenes, Aflatoxins and Related Mycotoxins*, Ministry for Foreign Affairs of Finland, Helsinki, 1986.
16 *Automatic Monitoring in Verification of Chemical Disarmament. Proceedings: The Finnish Project on Verification of Chemical Disarmament*, Ministry for Foreign Affairs of Finland, Helsinki, 1987.
17 *Handbook for the Investigation of the Use of Chemical or Biological Weapons*, Canada, 1985.
18 J.A. Dahlberg and I. B. Kihlman, *Acta Chem. Scand.*, 24 (1970) 644.
19 P. F. V. Ward and N. S. Huskisson, *Biochem. J.*, 113 (1969) 9.
20 E. Torres, J. F. Pereira and J. V. Oriente, *J. Sci. Food Agric.*, 42 (1988) 149.
21 *The Problem of Chemical and Biological Warfare*, Stockholm International Peace Research Institute, Almqvist and Wiksell, Stockholm 1971.
22 M. Basig, *Militartechnik*, 3 (1983) 153.
23 M. Sokolowski, *Wojsk. Przegl. Tech.*, 10 (1984) 435.
24 I. L. Knunyants (Editor), *Khemicheskiy Encyiklopedicheskiy Slowar*, Sovetskaya Entsiklopediya, Moscow, 1983.
25 M. F. Sartori, *Chem. Rev.*, 48 (1951) 236.
26 P. Bodoga, C. Marutoiu, A. Bresug, C. Sarbu and S. Gocan, *Rom. Pat.* 98 223 (1986).
27 R. L. Grob, *Khromatograficheskiy Analiz Okruzhayushcheiy Sredy*, Khimiya, Moscow, 1979.
28 B. Kolb (Editor), *Applied Headspace Gas Chromatography*, Heyden, London, Philadelphia, Rheine, 1980.
29 I. Sliwka, P. Rotocki, E. Broś and J. Lasa, *Chem. Anal. (Warsaw)*, 28 (1983) 3.
30 A. T. Pilipenko, M. V. Milyukin, A. S. Kuzemsa and F. M. Tulyupa, *Zh. Anal. Khim.*, 43 (1988) 136.
31 J. Drozd and J. Novak, *J. Chromatogr.*, 165 (1979) 141.
32 P. Peruzzi, D. Cursi and O. Griffini, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 450.
33 H. Binder, *J. Chromatogr.* 25 (1966) 189.
34 R. E. Majors, *LC · GC Int., Mag. Chromatogr. Sci.*, 2, No. 2 (1989) 12.
35 P. D. Fleur (Editor), *Accuracy in Trace Analysis, Sampling, Sample Handling, Analysis*, National Bureau of Standards, Washinghton, DC, 1976.
36 K. Beyermann, *Opredelenye Sledovych Kolichestv Organicheskich Veshchestv*, Mir, Moscow, 1987.
37 S. I. Muraveva, N. I. Kaznina and E. K. Prokhorova, *Handbook of Verification of Hazardous Substances*, Khimiya, Moscow, 1988.
38 J. Namieśnik, *Talanta*, 35 (1988) 567.
39 Yu. S. Drogov, *Zavod. Lab.*, 54 (1988) 3.
40 R. L. Erickson, R. N. Macnair, R. H. Brown and H. D. Hogan, *Anal. Chem.*, 44 (1972) 1040.
41 A. A. Casselman, N. C. C. Gibson and R. A. B. Bannard, *J. Chromatogr.*, 78 (1973) 317.
42 N. C. C. Gibson, A. A. Casselman and R. A. B. Bannard, *J. Chromatogr.*, 92 (1974) 162.
43 S. Sass, T. L. Fisher, R. J. Steger and G. A. Parker, *J. Chromatogr.*, 238 (1982) 445.
44 A. Kuessner, *Chromatographia*, 16 (1982) 207.
45 T. A. Misharina, I. L. Zhuravleva and R. V. Golovnya, *Zh. Anal. Khim.*, 42 (1987) 586.
46 P. W. Albro and L. Fishbein, *J. Chromatogr.*, 46 (1970) 202.
47 P. A. D'Agostino, A. S. Hansen, P. A. Lockwood and L. R. Provost, *J. Chromatogr.*, 347 (1985) 257.
48 H. M. Stahr, *J. Assoc. Off. Anal. Chem.*, 60 (1977) 1434.
49 Yu. S. Drugov and V. G. Berezkin, *Usp. Khim.*, 55 (1986) 999.
50 R. G. Lewis, A. R. Brown and M. D. Jackson, *Anal. Chem.*, 49 (1977) 1668.
51 C. L. Fraust and F. R. Hermann, *Am. Ind. Hyg. Assoc. J.*, 27 (1966) 68.
52 W. K. Fowler, C. H. Duffey and H. C. Miller, *Anal. Chem.*, 51 (1979) 2333.
53 A. Paudyn and J. C. van Loon, *Fresenius' Z. Anal. Chem.*, 325 (1986) 369.

54 J. P. Hendershott, *Am. Ind. Hyg. Assoc. J.*, 47 (1986) 742.
55 K. B. Sipponen, *J. Chromatogr.*, 389 (1987) 87.
56 M. Dressler, *J. Chromatogr.*, 165 (1979) 167.
57 K. G. Woolmington, *J. Appl. Chem.*, 11 (1961) 114.
58 R. E. Isbell, *Anal. Chem.*, 35 (1963) 255.
59 P. K. Basu, C.J. King and S. Lynn, *J. Chromatogr. Sci.*, 10 (1972) 479.
60 C. R. Schneider and H. Freund, *Anal. Chem.*, 34 (1962) 69.
61 W. Thain, *Monitoring Toxic Gases in the Atmosphere for Hygiene and Pollution Control*, Pergamon Press, Oxford, 1980.
62 I. R. Valenth, G. C.Carle and J. B. Phillips, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 269.
63 E. R. J. Wils and A. G. Hulst, *J. Chromatogr.*, 330 (1985) 379.
64 J. Paasivirta, H. Vihonen, J. Salovaara, J. Tarhanen, A. Veijanen, M. Lahtipera and R. Paukku, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 37.
65 S. I. Adam, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 369.
66 T. L. C. de Souza, *J. Chromatogr.*, 395 (1987) 413.
67 S. Zaromb, C. S. Woo, K. Quandt, L. M. Rice, A. Fermaint and L. J. Mitnaul, *J. Chromatogr.*, 439 (1988) 283.
68 H. C. de Bisschop, Y. G. Sergeant, P. P. Goethals and M. M. van Caekenberghe, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 141.
69 E. Kantolahti, R. Laitinen, T. Humppi and A. Laitinen, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 209.
70 J. Franzen, M. Weiss and A. G. London, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 231.
71 W. R. Hornbrook and R. H. Ode, *J. Chromatogr. Sci*, 25 (1987) 206.
72 G. L. Le Bel, D. T. Williams, G. Griffith and F. M. Benoit, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 241.
73 W. K. Fowler, J. E. Smith and H. C. Miller, *Anal. Chim. Acta*, 124 (1981) 225.
74 L. R. Marti, J. de Kanel and R. C. Dougherty, *Environ. Sci. Technol.*, 18 (1984) 973.
75 W. Pfannhauser and A. Thaller, *Fresenius' Z. Anal. Chem.*, 322 (1985) 220.
76 H. Ozawa and T. Tsukioka, *Anal. Chem.*, 59 (1987) 2914.
77 M. L. Shih and R. I. Ellin, *Anal. Lett.*, 19 (1986) 2197.
78 P. F. Blanchet, *J. Chromatogr.*, 179 (1979) 123.
79 R. W. Rosenthal, R. Proper and J. Epstein, *J. Phys. Chem.*, 60 (1956) 1596.
80 T. Vantiainen and P., Kauranen, *Anal. Chim. Acta*, 157 (1984) 91.
81 U. Niederschulte and K. Ballschmiter, *Fresenius' Z. Anal. Chem.*, 269 (1974) 360.
82 J. P. Ryan and J. S. Fritz, *J. Chromatogr. Sci.*, 16 (1978) 488.
83 V. Leoni, G. Puccetti, R. J. Colombo and A. M. D'Ovidio, *J. Chromatogr.*, 125 (1976) 399.
84 F. Mangani, G. Crescentini, P. Palma and F. Bruner, *J. Chromatogr.*, 452 (1988) 527.
85 C. Borra, A. di Corcia, M. Marchetti and R. Samperi, *Anal. Chem.*, 58 (1986) 2048.
86 K. Blau and G. S. King, *Handbook of Derivatives For Chromatography*, Heyden, London, 1978.
87 J. M. Rosenfeld, M. Mureika-Russell and A. Phatak, *J. Chromatogr.*, 283 (1984) 127.
88 E. Ebing and G. Hoffmann, *Fresenius' Z. Anal. Chem.*, 275 (1975) 11.
89 S. Bergek, in *Foreign Office Affairs Rep.*, C 40086-C2, Forsvarets Forskningsanstalt, Umeå, October 1978, p. 1.
90 M. C. Bowman, M. Beroza and K. R. Hill, *J. Assoc. Off Anal. Chem.*, 54 (1971) 346.
91 P. A. D'Agostino and L. R. Provost, *J. Chromatogr.*, 331 (1985) 47.
92 J. W. de Leeuw, W. B. de Leer, J. S. S. Damste and P. J. W. Schuyl, *Anal. Chem.*, 58 (1986) 1852.
93 Z. Jerzmanowska, *Substancje Roślinne. Metody Wyodrębniania*, Vol. I, PWN, Warsaw, 1967.
94 Z. Jerzmanowska, *Substancje Roślinne, Metody Wyordębniania*, Vol. II, PWN, Warsaw, 1970.
95 D. J. Sissons and G. M. Telling, *J. Chromatogr.*, 47 (1970) 328.
96 K. Sasaki, T. Suzuki and Y. Saito, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 460.
97 D. I. Zalutskaya and N. A. Salanshinskii, *Zh. Anal. Khim.*, 37 (1982) 494.
98 A. di Muccio, A. Ausili, I. Camoni, R. Dommarco, M. Rizzica and F. Vergori, *J. Chromatogr.*, 456 (1988) 149.

99 M. A. Klisenko and M. V. Pismennaya, *Zh. Anal. Khim.*, 43 (1988) 354.
100 J. L. Daft, *Bull. Environ. Contam. Toxicol.*, 30 (1983) 492.
101 B. Martinek and M. Walter, *Tluszcze Jadalne*, 17 (1973) 1.
102 K. A. McCully, *J. Assoc. Off. Anal. Chem.*, 55 (1972) 291.
103 I. Okuno and D. L. Meeker, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 49.
104 M. H. Yu and G. W. Miller, *Environ. Sci. Technol.*, 4 (1970) 492.
105 L. Leadbeater, G. L. Sainsbury and D. Utley, *Toxicol. Appl. Pharmacol.*, 25 (1973) 111.
106 H. M. Stevens, A. C. Moffat and J. V. Drayton, *Forensic Sci.*, 8 (1976) 131.
107 S. Sass and M. H. Stutz, *J. Chromatogr.*, 213 (1981) 173.
108 I. Okuno, D. L. Meeker and R. R. Felton, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 1102.
109 G. Machata and W. Vycudilik, *Arch. Belg. Med. Soc., Hyg., Med. Trav. Med. Leg., Suppl. 1st*, 1984, p. 53 (*Proceedings of the First World Congress: New Compounds in Biological and Chemical Warfare, Ghent, May 21–23, 1984*).
110 W. Vycudilik, *Forensic Sci. Int.*, 28 (1985) 131.
111 A. K. Singh, R. J. Zeleznikar, Jr., and L. R. Drewes, *J. Chromatogr.*, 324 (1985) 163.
112 H. C. Bisschop and E. Michiels, *Chromatographia*, 18 (1984) 433.
113 H. P. Benschop, E. C. Bijleveld, M. F. Otto, C. E. A. M. Degenhardt, H. P. M. van Helden and L. P. A. de Jong, *Anal. Biochem.*, 151 (1985) 242.
114 A. Heyndrickx, J. Cordonnier and A. de Bock, *Arch. Belg. Med. Soc., Hyg., Med. Trav. Med. Leg., Suppl. 1st*, 1984, p. 102 (*Proceedings of the First World Congress: New Compounds in Biological and Chemical Warfare, Ghent, May 21–23, 1984*).
115 G. Drasch, E. Kretschmer, G. Kauert and L. van Meyer, *J. Forensic Sci.*, 32 (1987) 1788.
116 R. W. Darr, T. L. Capson and F. D. Hileman, *Anal. Chem.*, 52 (1980) 1379.
117 A. Heyndrickx, H. de Puydt and J. Cordonnier, *Arch. Belg. Med. Soc., Hyg., Med. Trav. Med. Leg., Suppl. 1st*, 1984, p. 61 (*Proceedings of the First World Congress: New Compounds in Biological and Chemical Warfare, Ghent, May 21–23, 1984*).
118 E. R. J. Wils, A. G. Hulst, A. L. de Jong, A. Verweij and H. L. Boter, *J. Anal. Toxicol.*, 9 (1985) 254.
119 E. R. J. Wils, A. G. Hulst and J. van Laar, *J. Anal. Toxicol.*, 12 (1988) 15.
120 M. Stobiecki, *Wiad. Chem.*, 40 (1986) 495.
121 H. L. Kramer, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 1058.
122 A. C. Ray, L. O. Post and J. C. Reagor, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 19.
123 F. Geiss, *Fundamentals of Thin Layer Chromatography (Planar Chromatography)*, Hüthig, Heidelberg, Basle, New York, 1987.
124 E. Tyihak and E. Mincsovics, *J. Planar Chromatogr.*, 1 (1988) 6.
125 Z. Witkiewicz and J. Błądek, *J. Chromatogr.*, 373 (1986) 111.
126 Z. Witkiewicz and S. Rattay, *Farm. Pol.*, 39 (1983) 407.
127 E. Mincsovics, E. Tyihak and A. M. Siouffi, *J. Planar Chromatogr.*, 1 (1988) 141.
128 E. Tyihak, *J. Pharm. Biomed. Anal.*, 5 (1987) 191.
129 E. Tyihak, E. Mincsovics and H. Kalasz, *J. Chromatogr.*, 174 (1979) 75.
130 Z. Witkiewicz, *Nowe Kierunki w Chromatografii*, WNT, Warsaw, 1988.
131 E. Soczewiński, in R. E. Kaiser, (Editor), *Planar Chromatography*, Vol. 1, Hüthig, Heidelberg, 1986, p. 79.
132 G. Matysik and E. Soczewiński, *J. Chromatogr.*, 355 (1986) 363.
133 C. Marutoiu, M. Vlassa, C. Sarbu and S. Nagy, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 465.
134 A. Zlatkis and R. E. Kaiser (Editors), *HPTLC, High Performance Thin-Layer Chromatography*, Elsevier, Amsterdam, 1977.
135 J. Błądek, *J. Chromatogr.*, 405 (1987) 203.
136 J. Błądek, *J. Chromatogr.*, 437 (1988) 131.
137 J. Błądek and Z. Witkiewicz, *Pol. Pat.*, 144 560, 1988.
138 A. Stachlewska-Wróblowa, *Analiza Skazeń Chemicznych*, WAT, Warsaw 1981.
139 S. Franke, P. Franz, G. Grummer and W. Warnke, *Lehrbuch der Militarchemie*, Vol. 2, Militarverlag der Deutschen Demokratischen Republik, Berlin, 1977.
140 E. Soczewiński and Z. Suprynowicz (Editors), *Wspolczesne Kierunki Chromatografii*, UMCS, Lublin, 1986.
141 L. R. Snyder, *J. Chromatogr. Sci.*, 16 (1978) 223.
142 C. E. Mendoza, *J. Chromatogr.*, 78 (1973) 29.

143 H. Ackermann, *J. Chromatogr.*, 78 (1973) 39.
144 W. P. McKinley and S. L. Read, *J. Assoc. Off. Agric. Chem.*, 45 (1962) 467.
145 W. P. McKinley and P. S. Johal, *J. Assoc. Off. Agric. Chem.*, 46 (1963) 840.
146 C. E. Mendoza, P. J. Wales, H. A. McLeod and W. P. McKinley, *Analyst (London)*, 93 (1968) 34.
147 C. E. Mendoza, P. J. Wales, H. A. McLeod and W. P. McKinley, *Analyst (London)*, 93 (1968) 173.
148 C. E. Mendoza and J. B. Shields, *J. Chromatogr.*, 50 (1970) 92.
149 M. Bogusz and T. Borkowski, *Z. Rechtsmed.*, 68 (1971) 267.
150 S. Sandroni and H. Schlitt, *J. Chromatogr.*, 55 (1971) 385.
151 W. Winterlin, G. Walker and H. Frank, *J. Agric. Food Chem.*, 16 (1968) 808.
152 I. Sherma, *Anal. Methods Pestic. Plant Growth Regul.*, 14 (1986) 1.
153 A. Stachlewska-Wróblowa, *Biul. Wojsk. Akad. Tech.*, 33, No. 9 (1984) 45.
154 H. Kuhnen, *Mitt. Fraunhofer-Ges.*, 7 (1969) 2.
155 K. A. Jacobson and A. Patchornik, *J. Biochem. Biophys. Methods*, 8 (1983) 213.
156 A. Stachlewska-Wróblowa, *Biul. Wojsk. Akad. Tech.*, 27, No. 7 (1978) 135.
157 Z. Witkiewicz and S. Rattay, *Biul. Wojsk. Akad. Tech.*, 32, No. 2 (1983) 113.
158 B. Appler and K. Christmann, *J. Chromatogr.*, 264 (1983) 445.
159 S. Munavalli and M. Panella, *J. Chromatogr.*, 437 (1988) 423.
160 R. W. Pero and R. G. Owens, *Appl. Microbiol.*, 21 (1971) 546.
161 F. G. Stanford, *Analyst (London)*, 92 (1967) 64.
162 L. Fishbein and H. L. Falk, *Chromatogr. Rev.*, 11 (1969) 101.
163 A. Stachlewska-Wróblowa, *Chem. Anal. (Warsaw)*, 24 (1979) 1061.
164 A. Stachlewska-Wróblowa, *Biul. Wojsk. Inst. Chem. Radiom.*, No. 2 (7) (1980) 26.
165 W. D. Ludemann, M. H. Stutz and S. Sass, *Anal. Chem.*, 41 (1969) 679.
166 M. Haddadin, U. Khalidi, N. Turjuman and R. Ghougassian, *Anal. Chem.*, 46 (1974) 2072.
167 H. G. Eulenhofer, *J. Chromatogr.*, 36 (1968) 198.
168 H. Rosłonek, Z. Makles and M. Sokołowski, *Biul. Wojsk. Inst. Chem. Radiom.*, No. 1 (10) (1982) 22.
169 G. Szalontai, *J. Chromatogr.*, 124 (1976) 9.
170 V. L. McGuffin and M. Novotny, *Anal. Chem.*, 53 (1981) 946.
171 T. L. Chester, *Anal. Chem.*, 52 (1980) 1621.
172 P. J. Arpino, M. A. Baldwin and F. W. McLafferty, *Biomed. Mass Spectrom.*, 1 (1974) 80.
173 R. P. W. Scott, C. G. Scott, M. Munroer and J. Hess, Jr., *J. Chromatogr.*, 99 (1974) 395.
174 W. H. McFadden, H. L. Schwartz and S. Evans, *J. Chromatogr.*, 122 (1976) 389.
175 A. Farran, J. de Pablo and D. Barcelo, *J. Chromatogr.*, 455 (1988) 163.
176 F. W. Karasek and D. W. Denney, *Anal. Lett.*, 6 (1973) 993.
177 H. A. Moye, *J. Chromatogr. Sci.*, 13 (1975) 285.
178 S. H. Kim, F. W. Karasek and S. Rokushika, *Anal. Chem.*, 50 (1978) 152.
179 D. I. Carrol, I. Dzidic, R. N. Stillwell, M. G. Horning and E. C. Horning, *Anal. Chem.*, 46 (1974) 706.
180 S. R. Priebe and J. A. Howell, *J. Chromatogr.*, 324 (1985) 53.
181 K. B. Sipponen, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 269.
182 R. T. Krause, *J. Chromatogr.*, 185 (1979) 615.
183 W. A. Halang, R. Langlais and E. Kugler, *Anal. Chem.*, 50 (1978) 1829.
184 J. K. Baker and C. Y. Ma, *J. Chromatogr.*, 169 (1979) 107.
185 R. M. Smith, *Anal. Chem.*, 56 (1984) 256.
186 P. Kuronen, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 261.
187 K. A. Ramsteiner and W. D. Hormann, *J. Chromatogr.*, 104 (1982) 438.
188 C. D. Raghuveeran and R. C. Malhotra, *J. Chromatogr.*, 240 (1982) 243.
189 J. Krebs, R. J. Prime and K. Leung, *J. Can. Soc. Forensic Sci.*, 15 (1982) 29.
190 P. C. Bossle, J. J. Martin, E. W. Sarver and H. Z. Sommer, *J. Chromatogr.*, 283 (1984) 412.
191 R. Iadevaia, N. Aharonson and E. A. Woolson, *J. Assoc. Off Anal. Chem.*, 63 (1980) 22.
192 W. D. Spall, J. G. Lynn, J. L. Andersen, J. G. Valdez and L. R. Guerley, *Anal. Chem.*, 58 (1986) 1340.
193 D. M. Collins, J. P. Fawcett and C. G. Rammell, *Bull. Environ. Contam. Toxicol.*, 26 (1981) 669.
194 S. Lam and E. Grushka, *J. Chromatogr.*, 158 (1978) 207.
195 M. Hori, M. Suzuki, H. Kakinoki and Y. Kobaynashi, *Bunseki Kagaku*, 33 (1984) 430; *C.A.*, 101 (1984) 152708.

196 T. Someno, K. Katoh, K. Niijima and H. Miyazaki, *J. Chromatogr.*, 253 (1982) 81.
197 O. Gyllenhaal and J. Vessman, *J. Chromatogr.*, 435 (1985) 259.
198 A. Kuksis and P. Vishwakarma, *Can. J. Biochem. Physiol.*, 41 (1963) 2353.
199 A. Bevenue, J. N. Ogata and H. Beckman, *J. Chromatogr.*, 35 (1968) 17.
200 S. Sass and R. J. Steger, *J. Chromatogr.*, 238 (1982) 121.
201 S. Sass, L. Fisher, M. J. Jascot and J. Herban, *Anal. Chem.*, 43 (1971) 462.
202 I. Jane and B. B. Wheals, *J. Chromatogr.*, 70 (1972) 151.
203 I. Lindgren and B. Jansson, *J. Chromatogr.*, 106 (1975) 385.
204 K. Tesarik and K. Komarek, *Kapilarnye Kolonki w Gazowoi Khromatografii*, Mir, Moscow, 1987.
205 L. S. Ettre, *Introduction to Open Tubular Columns*, Perkin-Elmer, Norwalk, CT, 1983.
206 V. G. Berezkin and A. A. Korolev, *J. Chromatogr.*, 440 (1988) 323.
207 B. Xu and P. E. Vermeulen, *J. Chromatogr.*, 445 (1988) 1.
208 F. I. Onuska, R. I. Kominar and K. Terry, *J. Chromatogr. Sci.*, 21 (1983) 512.
209 H. J. Stan and H. Goebel, *J. Chromatogr.*, 314 (1984) 413.
210 G. Schomburg, H. Behlau, R. Dielmann and H. Husmann, *J. Chromatogr.*, 142 (1977) 87.
211 K. Grob and K. Grob, Jr., *J. High Resolut Chromatogr. Chromatogr. Commun.*, 1 (1978) 57.
212 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
213 H. van den Dool and P. D. Kratz, *J. Chromatogr.*, 11 (1963) 463.
214 J. N. Driscoll, *Int. Lab.*, No. 9 (1987) 68.
215 J. N. Driscoll, *J. Chromatogr. Sci.*, 23 (1985) 488.
216 V. Danes, *Czech. Pat.*, 184 565 (1980).
217 G. E. Spangler and D. N. Campbell, *Eur. Pat. Appl.*, EP 219 602 (1987).
218 J. C. Schmidt, D. N. Campbell and S. C. Goetz, *Eur. Pat. Appl.*, EP 221 382 (1987).
219 Y.-C. Yang, L. L. Szafraniec, W. T. Beadury and J. R. Ward, *J. Org. Chem.*, 52 (1987) 1637.
220 R. L. Holmstead and J. E. Casida, *J. Assoc. Off. Anal. Chem.*, 57 (1974) 1050.
221 H. J. Stan, *Fresenius' Z. Anal. Chem.*, 287 (1977) 104.
222 K. L. Busch, M. M. Bursey, J. Hass and G. W. Sovocool, *Appl. Spectrosc.*, 32 (1978) 388.
223 S. Sass, T. L. Fisher, *Org. Mass Spectrom.*, 14 (1979) 257.
224 P. A. Cload and D. W. Hutchinson, *Org. Mass Spectrom.*, 18 (1983) 57.
225 R. M. Martz, D. J. Reutter and L. D. Lasswell, *J. Forensic Sci.*, 28 (1983) 200.
226 M. Basig, *Militartechnik*, 4 (1983) 200.
227 P. A. D'Agostino and L. R. Provost, *Biomed. Environ. Mass Spectrom.*, 13 (1986) 231.
228 A. Hesso and R. Kostiainen, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 257.
229 U. M. Lakkisto, in *Proceedings of the 2nd International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 15–19 June 1986*, National Defence Research Institute, Umeå, 1986, p. 245.
230 S. Sass, S. Pinsky and M. R. Barnes, *Chem. Styst. Lab. CRDL Tech. Memo*, 62–2, Chemical Systems Laboratory, Aberdeen Proving Ground, MD, 1963.
231 S. S. Brody and J. E. Chaney, *J. Gas Chromatogr.*, 4 (1966) 42.
232 T. L. Fisher, R. J. Steger, G. A. Parker and S. Sass, *Report 1976*, EC-TR-76080; Order No. AD-B015464, 25 pp.; available NTIS, From Government Reports Announce, Index (US) 78 (1978) 140; *C.A.*, 90 (1979) 17157.
233 M. Vujadinovic, Z. Radic and S. Jovanovic, *Naučnotehnički Pregled*, 31 (1981) 33; *C.A.*, 97 (1982) 18503.
234 D. N. Tripathi, M. P. Kaushik and A. Bhattacharya, *J. Can. Soc. Forensic Sci.*, 20 (1987) 151.
235 W. H. Griest and T. W. Martin. *J. Chromatogr.*, 192 (1980) 173.
236 T. Du, *Int. J. Environ. Anal. Chem.*, 27 (1986) 151.
237 P. A. D'Agostino, L. R. Provost and J. Visentini, *J. Chromatogr.*, 402 (1987) 221.
238 S. Sass and G. A. Parker, *J. Chromatogr.*, 189 (1980) 331.
239 C. E. A. M. Degenhardt, G. R. van den Berg, L. P. A. de Jong, H. P. Benschop, J. van Genderen and D. van de Meent, *J. Am. Chem. Soc.*, 108 (1986) 8290.
240 C. E. A. M. Degenhardt, A. Verweij and H. P. Benschop *Int. J. Environ. Anal. Chem.*, 30 (1987) 15.
241 A. Verweij, E. Burghardt and A. W. Koonings, *J. Chromatogr.*, 54 (1971) 151.
242 S. Okonek, *Arch. Toxikol.*, 29 (1972) 255.
243 C. J. Briggs and K. J. Simons, *J. Chromatogr.*, 257 (1983) 132.

244 S. H. Sterri, G. Valdal, S. Lyngaas, E. Odden, D. Malthe-Sorenssen and F. Fonnum, *Biochem. Pharmacol.*, 32 (1983) 1941.
245 O. Beck. B. Holmstedt, J. Lundin, G. Lundgren and J. Stantesson, *Fundam. Appl. Toxicol.*, 1 (1981) 148; *C.A.*, 96 (1982) 137216.
246 H. P. Benschop, C. A. G. Konings and L. P. A. de Jong, *J. Am. Chem. Soc.*, 103 (1981) 4260.
247 L. P. A., de Jong, E. C. Bijlevald, C. van Dijk and H. P. Benschop, *Int. J. Environ. Anal Chem.*, 29 (1987) 179.
248 K. Kosmala and T. Lis, *Biul. Wojsk. Inst. Chem. Radiom.*, No. 2 (7) (1980) 33.
249 G. Machata, *Arch. Kriminol.*, 160 (1977) 151.
250 D. K. Rohrbaugh, Y. C. Yang and J. R. Ward, *J. Chromatogr.*, 447 (1988) 165.
251 P. A. D'Agostino and L. R. Provost, *J. Chromatogr.*, 436 (1988) 399.
252 W. Vycudilik, *Forensic Sci. Int.*, 28 (1985) 131.
253 W. Vycudilik, *Forensic Sci. Int.*, 35 (1987) 67.
254 F. Ellingsen and P. J. Karlsen, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 101.
255 K. Schoene and J. Steinhanses, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 215.
256 R. M. Black and R. W. Read, *J. Chromatogr.*, 449 (1988) 261.
257 C. Różycki, M. Sokołowski and Z. Makles, *Biul. Wojsk. Inst. Chem. Radiom.*, No. 1 (9) (1981) 37.
258 J. D. Ramsey and T. D. Lee, *J. Chromatogr.*, 184 (1980) 185.
259 E. N. Zerba and M. A. Ruveda, *J. Chromatogr.*, 68 (1972) 245.
260 C. J. Stahl, B. C. Young, R. J. Brown and C. A. A. Sworth, *J. Forensic. Sci.*, 13 (1968) 442.
261 H. W. Avdovich, A. By, J. C. Ethier and G. A. Neville, *Can. Soc. Forensic. J.*, 14 (1981) 172.
262 D. N. Tripathi, R. C. Malhotra and A. Bhattacharya, *J. Chromatogr.*, 315 (1984) 417.
263 K. E. Ferelew, R. H. Orcutt and A. N. Hagardorn, *J. Forensic Sci.*, 31 (1986) 658.
264 J. Nowicki, *J. Forensic Sci.*, 27 (1982) 704.
265 J. G. Merck-Luengo, *Pestic. Chem., Proc. Int. Congr. Pestic. Chem., 2nd*, 4 (1971) 227.
266 D. Revuelta, G. Revuelta and F. Armisen, *An. Quim.*, 71 (1975) 503; *C.A.*, 83 (1975) 162090.
267 G. Pallotti, B. Bencivenga, A. Consolino, S. de Vincenzi, G. Filiaci and A. Palmioli, *Boll. Chim. Unione Ital. Lab. Prov., Parte Sci.*, 33 (1982) 61.
268 N. Saito, Y. Ogino and M. Nagao, *Okayama-ken Kankyo Hoken Senta Nempo*, 3 (1979) 173; *C.A.* 92 (1980) 157289.
269 K. Hasegawa, S. Itoh and S. Naito, *Kanagawa-ken Eisei Kenkyusko, Kenkyu Hokoku*, 13 (1983) 19; *C.A.*, 101 (1984) 197769.
270 J. L. Daft, *J. Agric. Food Chem.*, 33 (1985) 563.
271 J. L. Daft, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 734.
272 R. D. Lingg, R. G. Melton and F. C. Kopfler, *J. Am. Water Works Assoc.*, 69 (1977) 605.
273 K. Jiang, Z. Kang. X. Zhu, G. Zhao and Y. Bian, *Huanjing Kexue Xuebao*, 2 (1982) 262; *C.A.*, 97 (1982) 187985.
274 J. Burke and L. Johnson, *J. Assoc. Off. Agric. Chem.* 45 (1962) 348.
275 B. Berck, *J. Agric. Food Chem.*, 113 (1965) 373.
276 A. Yasuhara, M. Morita and K. Fuwa, *J. Chromatogr.*, 328 (1985) 35.
277 T. A. Kozeiko, M. A. Aizenshtadt, E. D. Maktaz and V. S. Kasman, *Gig. Sanit.*, 7 (1987) 41; *C.A.*, 107 (1987) 204787.
278 J. L. Daft, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 228.
279 E. P. Cheremukhin and Z. F. Kanivets, *Avtomatich. Analitich. Kontrol v Proiz-ve Ioda i Broma, Moscow*, 1978; *Ref. Zh. Khim.*, (1979) 11 G 235; *C.A.*, 91 (1979) 116923.
280 B. Berck, *J. Chromatogr. Sci.*, 13 (1975) 256.
281 J. Kanazawa, *Agric. Biol. Chem.*, 27 (1963) 159; *C.A.*, 59 (1963) 5709.
282 J. E. Peterson, *Bull. Environ. Contam. Toxicol.*, 13 (1975) 751.
283 J. Tannock, *Rhod. J. Agric. Res.*, 13 (1975) 55; *C.A.*, 83 (1975) 109303.
284 A. C. Ray, L. O. Post, T. P. Hewlett and J. C. Reagor, *Vet. Hum. Toxicol.*, 23 (1981) 418; *C.A.*, 96 (1982) 47045.
285 S. G. Heuser and K. A. Scudamore, *Chem. Ind. (London)*, 29 (1967) 1557.
286 C. J. Soderquist, D. G. Crosby and J. B. Bowers, *Anal. Chem.*, 46 (1974) 155.

287 F. Fukui, *Talanta*, 28 (1981) 402.
288 B. Beckermann, *Anal. Chim. Acta*, 135 (1982) 77.
289 K. Dix, C. J. Cappon and T. Y. Toribara, *J. Chromatogr. Sci.*, 25 (1987) 164.
290 H. M. Stahr and D. L. Meeker, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 49.
291 D. T. Canvin, *Can. J. Biochem.*, 43 (1965) 1281.
292 H. Gershon and J. A. A. Renwick, *J. Chromatogr.*, 20 (1965) 134.
293 H. Gershon and J. A. A. Renwick, *J. Chromatogr.*, 28 (1967) 399.
294 M. Kotakemori, T. Sawada, H. Nishizawa and K. Obayashi, *Bunseki Kagaku*, 18 (1969) 392; *C.A.*, 71 (1969) 2446.
295 H. M. Stahr, W. B. Buck and P. F. Ross, *J. Assoc. Off. Anal. Chem.*, 57 (1974) 405 and 1230.
296 I. Okuno, G. E. Connolly, P. J. Savarie and C. P. Breidenstein, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 549.
297 H. H. Casper, T. L. McMahon and G. D. Paulson, *J. Assoc. Off Anal. Chem.*, 68 (1985) 722.
298 J. J. L. Hoogenboom and C. G. Rammell, *J. Anal. Toxicol.*, 11 (1987) 140.
299 H. D. Durst, M. Milano, E. J. Kikta, S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
300 Y. Hirose, I. Morimoto, T. Okitsu, N. Maeda and S. Kanno, *Eisei Kagaku*, 34 (1988) 65; *C.A.*, 108 (1988) 209870.
301 G. Gao, Z. Huang, J. Zhao and C. Zhao, *Huanjing Kexue*, 4 (1983) 43; *C.A.*, 99 (1983) 58580.
302 Y. Hanoi, T. Katou and T. Iizuka, *Yokohama Kokuritsu Daigaku Kankyo Kagaku Kenkyu Senta Kiyo*, 10 (1983) 31; *C.A.*, 100 (1984) 90504.
303 M. Paobo, M. M. Birky and S. E. Womble, *J. Combust. Toxicol.*, 6 (1979) 99.
304 E. M. Cumming and B. E. Frost, *Res. Discl.*, 135 (1975) 37; *C.A.*, 83 (1975) 187781.
305 V. G. Berezkin, *Gazo-Zhindko-Twierdophaznaia-Khromatografia*, Khimiya, Moscow, 1986.
306 A. L. Myerson and J. J. Chludzinski, Jr., *J. Chromatogr. Sci.*, 13 (1975) 554.
307 A. A. Karnishin and B. M. Bulybin, *Zavod. Lab.*, 39 (1973) 1063.
308 S. Araki, T. Kato and T. Atobe, *Bunseki Kagaku*, 12 (1963) 450; *C.A.*, 59 (1963) 10762.
309 J. Furuno, N. Sugawane and Y. Irizawa, *Igaku To Seibutsugaku*, 91 (1975) 109; *C.A.*, 85 (1976) 104749.
310 S. A. Liebman, D. H. Ahlstrom and C. I. Sanders, *Am. Lab.*, 7 (1975) 21.
311 D. P. Manka, *Instrum. Technol.*, 22 (1975) 45.
312 Yu. N. Ilin, S.A. Galushkina and L. F. Vasyutin, *Koks Khim.*, 2 (1977) 32.
313 I. A. Ustinovskaya, L. I. Kizilowa and L. Yu. Gavrilina, *Izv. Sib. Otd. Akad. Nauk SSSR, Ser. Khim. Nauk*, 2 (1977) 115.
314 S. J. Gentry and P. T. Walsh, *Am. Ind. Hyg. Assoc. J.*, 48 (1987) 287.
315 V. K. Steba, V. I. Zrazherskii, V. D. Parkhomenko and A. A. Pivovarov, *Khim. Prom., Ser. Metody Anal. Kontrolya Kach. Prod. Khim. Promsti.*, 11 (1979) 6.
316 W. F. Wilhite and O. L. Hollis, *J. Gas Chromatogr.*, 6 (1968) 84.
317 H. P. Schuchmann and K. J. Laidler, *J. Air Pollut. Control. Assoc.*, 22 (1972) 52.
318 N. Fukuda, H. Itoh, A. Tsukamoto and H. Tamari, *Bunseki Kagaku*, 28 (1979) 569; *C.A.*, 92 (1980) 27645.
319 H. Kachi and Y. Nakamno, Iidosha Gijutsuhai Ronbunshu, 20 (1980) 64; *C.A.*, 93 (1980) 244576.
320 S. M. Banna and M. C. Branch, *Combust. Sci. Technol.*, 24 (1980) 15.
321 N. M. Nikolaeva and A. G. Fonkich, *Gazov. Khromatogr.*, 3 (1965) 149.
322 E. Urbas and E. Kullik, *Eesti NSV Tead. Akad. Toim., Keem.*, 28 (1979) 124; *C.A.*, 91 (1979) 128166.
323 J. M. Murrell, *Fire Mater.*, 10 (1986) 57.
324 E. Urbas and E. Kullik, *J. Chromatogr.*, 137 (1977) 210.
325 P. Ingham, *J. Appl. Polym. Sci.*, 15 (1971) 3205.
326 W. E. Coleman, R. D. Lings and R. G. Melton, *Identif. Anal. Org. Pollut. Water*, (1976) 305; *C.A.*, 86 (1977) 126962.
327 M. Donike, *Z. Naturforsch.*, 28 (1973) 533.
328 M. Donike, *Mitteilungsbl. GDCh-Fachgruppe Lebensmittelchem. Gerichtl. Chem.*, 28 (1974) 46.
329 F. A. Gunther, A. Lopez-Roman and J. H. Barkley, *J. Agric. Food Chem.*, 19 (1971) 508.
330 T. Chikamoto and T. Maitani, *Kyoto-fu Eisei Kogai Kenkyusho Nempo*, 29 (1984) 147; *C.A.*, 103 (1985) 36248.
331 F. Gadel and A. Bruchet, *Water Res.*, 21 (1987) 1195.
332 K. D. Brunnemann, L. Yu and D. Hoffmann, *J. Anal. Toxicol.*, 1 (1977) 38.
333 J. Enqvist, M. Kokko, M. Pajarinen and U. M. Lakkisto, *Kem. Kemi*, 12 (1985) 793; *C.A.*, 105 (1986) 107661.

334 J. C. Valentour, V. Aggarwal and I. Sunshine, *Anal. Chem.*, 46 (1974) 924.
335 P. N. Brown, G. G. Jayson and M. C. Wilkinson, *Chromatographia*, 21 (1986) 161.
336 H. Runge, *Fresenius' Z. Anal. Chem.*, 189 (1962) 111.
337 A. Fish, N. H. Franklin and R. T. Pollard, *J. Appl. Chem.*, 13 (1963) 506.
338 J. Wilke, A. Losse and H. Sackmann, *J. Chromatogr.*, 18 (1965) 482.
339 L. J. Priestley, Jr., F. E. Critchfield, N. H. Ketcham and J. D. Cavender, *Anal. Chem.*, 37 (1965) 70.
340 A. L. Linch, S. S. Lord, Jr., K. A. Kubitz and M. R. de Brunner, *Am. Ind. Hyg. Assoc. J.*, 26 (1965) 465.
341 H. Sackmann, A. Losse and J. Wilke, *Abh. Dtsch. Akad. Wiss. Berlin, Kl. Chem. Geol. Biol.*, 2 (1966) 269.
342 S. T. Sie, J. P. Bleumer and G. W. A. Rijnders, *Sep. Sci.*, 3 (1968) 165.
343 A. Kuessner, *Anal. Chem.*, 204 (1981) 159.
344 P. Lacaze, *Chromatographia*, 12 (1979) 803.
345 J. E. Lovelock, R. J. Maggs and E. R. Adlard, *Anal. Chem.*, 43 (1971) 1962.
346 D. Lillian and H. B. Singh, *Anal. Chem.*, 46 (1974) 1060.
347 H. B. Singh, D. Lillian and A. Appleby, *Anal. Chem.*, 47 (1975) 860.
348 D. Lillian, H. B. Singh and A. Appleby, *J. Air Pollut. Control Assoc.*, 26 (1976) 141.
349 D. Reichert, U. Spengler and D. Henschler, *J. Chromatogr.*, 179 (1979) 181.
350 P. Lacaze, *Chromatographia*, 6 (1973) 32.
351 R. Jeltes, E. Burghardt and J. Breman, *Br. J. Ind. Med.*, 28 (1971) 96.
352 G. G. Esposito, D. Lillian, G. E. Podolak and R. M. Tuggle, *Anal. Chem.*, 49 (1977) 1774.
353 M. Drygas, *Organika*, (1978) 121.
354 R. J. Graham and F. D. Stevenson, *J. Chromatogr.*, 47 (1970) 555.
355 A. Baiker, H. Geisser and W. Richan, *J. Chromatogr.*, 147 (1978) 453.
356 G. Parissakis, D. Vrant-Piscou and J. Z. Kontoyannakos, *Fresenius' Z. Anal. Chem.*, 254 (1971) 188.
357 N. Kh. Agliulov, I. A. Zelgaev, M. V. Zueva, V. V. Luchinkin, L. G. Nikolaeva and I. A. Feshchenko, *Tr. Khim. Khim. Technol.*, 3 (1973) 66.
358 M. V. Zueva, V. Ya. Dudorov, N. Kh. Agliulov and Yu. M. Salganskii, *Zh. Anal. Khim.*, 31 (1976) 185.
359 G. G. Devyatykh, V. A. Krylov, Yu. M. Salganskii, T. N. Radkevich and A. E. Nikolaev, *Zh. Anal. Khim.*, 32 (1977) 2372.
360 V. A. Krylov, S. G. Krasnowa and Yu. M. Salganskii, in G. G. Devyatykh (Editor), *Poluch. Anal. Veshchestv Osoboi Chistoty, [Dokl. Vses. Konf.]*, 5th 1976 (Pub. 1978), Nauka, Moscow, 1978, p. 190; *C.A.*, 911 (1979) 116740.
361 L. G. Nikolayeva, V. A. Krylov, Z. K. Borisova, I. L. Agafonov and Yu. M. Salganskii, *Zh. Anal. Khim.*, 37 (1982) 2178.
362 D. Kiraly and A. Peter, *Acta Phys. Chem.*, 23 (1977) 313.
363 W. A. Koenig, E. Steinback and K. Ernst, *J. Chromatogr.*, 301 (1984) 129.
364 W. A. Koenig, O. Gyllenhaal and J. Vessman, *J. Chromatogr.*, 356 (1986) 354.
365 O. Gyllenhaal, *Analyst (London)*, 108 (1983) 978.
366 V. G. Berezkin and V. N. Retunskii, *Zh. Anal. Khim.*, 43 (1988) 166.
367 G. D. Sides, H. C. Miller, C. R. Lishawa and K. A. Kuhn, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 39.
368 G. D. Sides, C. R. Lishawa, D. W. Mason and B. W. Reuben, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 113.
369 F. Z. Grandi, R. Basei and L. Szpyrkowicz, *Ann. Chim.*, 77 (1987) 899.
370 E. Dahlgren, in *Proceedings of the International Symposium on Protection Against Chemical Warfare Agents, Stockholm, Sweden, 6–9 June 1983*, National Defence Research Institute, Umeå, 1983, p. 77.
371 T. Otogawa, J. R. Stetter and S. Zaromb, *J. Chromatogr.*, 360 (1986) 252.
372 J. Jerpe and A. Davis, *J. Chromatogr. Sci.*, 25 (1987) 154.
373 W. C. Kuster, P. D. Goldan and F. C. Fehsenfeld, *J. Chromatogr.*, 205 (1981) 271.
374 J. Namiesnik, T. Gorecki, E. Kozłowski, L. Torres and J. Mathieu, *Sci. Total Environ.*, 38 (1984) 225.
375 K. Goergens, H. Engels, N. Dammann and M. Lippelt, *Ger. Pat.*, DE 3 710 779, 1988.
376 J. L. Daft, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 748.
377 P. Subra, M.-C. Hennion, R. Rosset and R. W. Frei, *J. Chromatogr.*, 456 (1988) 121.

378 J. Curvers, T. Noij, C. Cramers and J. Rijks, *Chromatographia*, 19 (1985) 225.
379 B. Kolb, P. Pospisil and M. Auer, *Chromatographia*, 19 (1985) 113.
380 A. Bianchi, M. S. Varney and J. Philips, *J. Chromatogr.*, 467 (1989) 111.
381 C. H. Lochmuller, *J. Chromatogr. Sci.*, 25 (1987) 583.
382 C. F. Poole and S. K. Poole, *Anal. Chim. Acta*, 216 (1989) 109.
383 M. Warner, *Anal. Chem.*, 59 (1987) 1403A.
384 V. G. Berezkin and E. N. Viktorova, *Zh. Anal. Khim.*, 43 (1988) 2099.
385 P. A. D'Agostino and L. R. Provost, *Biomed. Environ. Mass. Spectrom.*, 15 (1988) 553.
386 P. A. D'Agostino, L. R. Provost and K. M. Looye, *J. Chromatogr.*, 465 (1989) 271.
387 H. C. Thompson, Jr., W. M. Blakemore, D. M. Nestorick, J. P. Freeman and D. W. Miller, *J. Chromatogr.*, 467 (1989) 159.
388 J. A. Tornes and B. A. Johnsen, *J. Chromatogr.*, 467 (1989) 129.
389 M. Ranny, *Thin-Layer Chromatography with Flame Ionization Detection*, Reidel, Dordrecht, Boston, Lancaster, Tokyo, 1986.
390 V. G. Berezkin and V. S. Gavrichev, *Usp. Khim.*, 58 (1989) 334.
391 S. M. Fields and K. Grolimund, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 727.
392 K. D. Bartle, M. P. Burke, A. A. Clifford, I. L. Davies, J. P. Kithinji, M. W. Raynor, G. F. Shilstone and A. Williams, *Eur. Chromatogr. News*, 2, No. 5 (1988) 12.
393 J. D. Pinkston, *Trends Anal. Chem.*, 7 (1988) 154.
394 R. D. Smith, B. W. Wright and C. R. Yonker, *Anal. Chem.*, 60 (1988) 1323A.
395 P. R. Griffiths, *Anal. Chem.*, 60 (1988) 593A.
396 H. Kallio and T. Shibamoto, *J. Chromatogr.*, 454 (1988) 392.
397 K. Schoene, J. Steinhanses and A. Koenig, *J. Chromatogr.*, 455 (1988) 67.
398 J. P. Duguet, C. Anselme and J. Mallevialle, *Water Supply*, 6 (1988) 253; *C.A.*, 110 (1989) 44636.
399 Y. C. Yang, D. K. Rohrbaugh and R. J. Ward, *Gov. Rep. Announce. (U.S.)*, 88(12) (1988), Report 1987, Abstr. No. 831 374; C.A., 110 (1989) 62935g.
400 S. H. Hoke and M. L. Shih, *Gov. Rep. Announce. (U.S.)*, 88(14) (1988), Report 1987, Abstr. No. 836 777; C.A., 110 (1989) 82155f.
401 J. R. Hancock and R. P. Hicken, *Gov. Rep. Announce. (U.S.)*, 88(15) (1988), Report 1987, Abstr. No. 838 858; C.A., 110 (1989) 101404e.
402 G. D. Byrd, L. T. Sniegoski and E. White, *Gov. Rep. Announce. (U.S.)*, 88(16) (1988) Report 1987, Abstr. No. 842 099; C.A., 110 (1989) 70602m.
403 M. W. Ellzy, F. E. Ferguson and L. G. Janes, *Gov. Rep. Announce. (U.S.)*, 89(7) (1989), Report 1988, Abstr. No. 916 035; C.A., 111 (1989) 95949p.
404 B.S. Goodell, *Wood Fiber Sci.*, 21 (1989) 37.
405 J. S. Little, C. A. Broomfield, M. K. Fox-Talbot, L. J. Boucher, B. MacIver and D. E. Lenz, *Biochem. Pharmacol.*, 38 (1989) 23.
406 J. L. Daft, *J. Agric. Food Chem.*, 37 (1989) 560.
407 P. E. F. Zoun and Th. J. Spierenburg, *J. Chromatogr.*, 462 (1989) 448.
408 M. Maruo, N. Iiirayama, I. Wada and T. Kuwamoto, *J. Chromatogr.*, 466 (1989) 379.
409 M. Zief, *Solid-Phase Extraction for Sample Preparation*, J. T. Baker, Phillipsburgh, NJ, 1989.
410 H. Ozawa and T. Tsukoka, *J. Chromatogr.*, 473 (1989) 251.
411 J. Vinuesa, J. C. M. Cortes, C. I. Canas and G. F. Perez, *J. Chromatogr.*, 472 (1989) 365.
412 W. K. Fowler and J. E. Smith, Jr., *J. Chromatogr.*, 478 (1989) 51.
413 A. P. Brestkin, E. B. Nikolskaya, L. P. Kuznietzova, B. M. Kostrova and N. A. Tulina, *Zh. Anal. Khim.*, 44 (1989) 1666.

*Journal of Chromatography*, 503 (1990) 359–368
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 187

# Isotope dilution gas chromatography–mass spectrometry in the determination of benzene, toluene, styrene and acrylonitrile in mainstream cigarette smoke

G.D. BYRD*, K.W. FOWLER, R.D. HICKS, M.E. LOVETTE and M.F. BORGERDING
*R. J. Reynolds Tobacco Company, Bowman Gray Technical Center, Winston-Salem, NC 27102 (U.S.A.)*
(First received August 4th, 1989; revised manuscript received December 4th, 1989)

SUMMARY

A cryogenic trapping method with isotope dilution gas chromatography–mass spectrometry analysis has been developed for the determination of benzene, toluene, styrene and acrylonitrile in mainstream vapor phase cigarette smoke. The method is simple, direct, and quantitative. Vapor phase samples are collected cryogenically in a series of four traps following removal of the particulate phase with a Cambridge filter pad. For all four analytes, 75–85% of the total amounts recovered were found in the initial trap and less than 1% in the final trap. Assessment of instrumental precision by multiple injections of a sample gave relative standard deviations of less than 2%. Linear calibration for all analytes over the analysis range gave an $r^2$ value greater than 0.99 with average relative standard deviations at the mean ranging from 1.4 to 8.2%. The cigarettes analyzed include a reference cigarette (Kentucky 1R4F), a commercial ultra-low "tar" mentholated cigarette, and two cigarettes that heat but do not burn tobacco. The values determined for the four analytes in the 1R4F samples are comparable to reported values of similar cigarettes. The cigarettes which heat rather than burn tobacco yield less of all four analytes compared to the other cigarettes in the study.

## INTRODUCTION

The determination of volatile organic compounds in cigarette smoke is challenging due to the complexity of the smoke matrix[1]. With the advent of low and ultra-low tar brands, yields of volatile organics are generally on the order of micrograms per cigarette. The recent development of a cigarette brand that heats but does not burn tobacco[2] presents a different type of smoke matrix for which precise and accurate smoke composition data are also useful.

The determination of minor smoke components often requires a concentration step prior to instrumental analysis because cigarette smoke is a dilute, complex mixture consisting mainly of air[1]. Two approaches widely used for this purpose are

0021-9673/90/$03.50 

cryogenic trapping[3–7] and sorption–desorption methods with a solid substrate such as Tenax[8–10].

Cryogenic trapping has been used historically in the analysis of vapor phase cigarette smoke to collect samples under conditions of low reactivity[3–5]. This minimizes sample degradation which is an important concern for quantitative measurements of analytes at low concentrations. Cryogenic methods using two different types of cold traps have been reported[6,7]. A glass trap submerged in liquid nitrogen has been used to collect whole smoke from a domestic filter blend cigarette with quantitative analysis by gas chromatography (GC)[6]. A recent report has demontrated that vapor phase smoke can also be sampled directly from individual puffs and trapped on a cold capillary column for subsequent GC–mass spectrometric (MS) analysis[7].

Vapor phase components from smoke can be trapped on Tenax and thermally desorbed onto a gas chromatograph for analysis[8]; however, Tenax has a low loading capacity for highly volatile organics[9] and some breakthrough has been noted of highly volatile smoke components from the Tenax trap from cigarettes that have higher tar yields[8]. In addition, an undesirable high-temperature desorption step is required to release the analytes from Tenax. Tenax can contribute background response to some analytes of interest[10] which ultimately affects both precision and accuracy.

Our objectives in this work were to develop a method that could determine selected smoke components from different types of cigarettes and which could accommodate a wide range of analyte concentrations. To meet these objectives we combined cryogenic trapping of cigarette smoke with isotope dilution GC–MS. Cigarette smoke is trapped in methanol at $-70°C$ and samples are analyzed without additonal concentration or purification steps. The simplicity of the procedure favors quantitative analysis because potential losses from chemical reaction, analyte decomposition, and non-quantitative transfer during extensive chemical fractionation are minimized. Operating the mass spectrometer in the selected-ion monitoring mode virtually eleminates background contributions and the use of isotopically labelled analogs as internal standards (isotope dilution) provides a more precise and accurate method for quantifying the trapped analytes than external standard or conventional internal standard methods[11]. Isotopically labelled analogs compensate for potential losses during sample transfer and for instrumental variability because they have physical, chromatographic, and mass spectral properties that are nearly identical to those of the analytes.

In this study the method is applied to low-tar and ultra-low-tar cigarettes, and to cigarettes that heat but do not burn tobacco. The analytes determined are acrylonitrile, benzene, toluene and styrene. These compounds are all associated with the vapor phase of cigarette smoke and have been previously reported in cigarette smoke[11–13].

## EXPERIMENTAL

### *Cigarettes*

Four different cigarettes were analyzed in this study. The cigarettes included the 1R4F reference cigarette produced by the Tobacco and Health Research Institute (Lexington, KY, U.S.A.) and a commercial ultra-low-tar mentholated brand (ciga-

rette A). Two cigarettes that heat rather than burn tobacco were analyzed, one regular (cigarette B) and one mentholated (cigarette C).

*Chemicals*

Acrylonitrile, benzene, toluene and styrene were obtained from Aldrich (Milwaukee, WI, U.S.A.). [$^2H_8$]Styrene and [$^2H_3$]acrylonitrile were obtained from Cambridge Isotope Labs. (Woburn, MA, U.S.A.). [$^2H_6$]Benzene and [$^2H_8$]toluene were obtained from MSD Isotopes (Montreal, Canada). The purities of all isotopically labelled materials were 98 atom% $^2H$ or greater. Methanol was high-purity solvent grade obtained from American Burdick and Jackson (Muskegon, MI, U.S.A.).

*Solutions*

A primary stock solution of each analyte was prepared by accurately weighing into a 10-ml volumetric flask 100 $\mu$l of the neat analyte. Each solution was diluted to the mark with methanol and mixed well. A secondary stock solution was prepared by adding the following volumes from the primary stock solutions into one 10-ml volumetric flask: 0.400 ml acrylonitrile, 1.00 ml benzene, 1.00 ml toluene and 0.100 ml styrene. The solution was diluted to the mark with methanol and mixed well.

Stock solutions of [$^2H_3$]acrylonitrile and [$^2H_8$]styrene were prepared by weighing accurately into two 10-ml volumetric flasks 100 $\mu$l of each neat material, respectively. Each solution was diluted to the mark with methanol and mixed well. An internal standard spiking solution was prepared by adding 1.00 ml of the [$^2H_3$]-acrylonitrile stock solution and 0.400 ml of the [$^2H_8$]styrene stock solution to a 10-ml volumetric flask and by accurately weighing 50 $\mu$l of [$^2H_6$]benzene and 50 $\mu$l of [$^2H_8$]toluene to the flask. The solution was diluted to the mark with methanol and mixed well.

Four standard solutions were prepared by adding 100, 500, 1500 and 3000 $\mu$l of the secondary stock solution to four respective 10-ml volumetric flasks. A volume of 100 $\mu$l of the internal standard spiking solution was added to each flask. The solutions were diluted to the mark with methanol and mixed well. All solutions were stored at 4°C and allowed to warm to room temperature before use.

*Smoke generation and collection apparatus*

Mainstream vapor phase smoke was isolated by using the apparatus shown in Fig. 1. Cigarettes were smoked on a Model RM20/CS 20-port Heinrich Borgwaldt

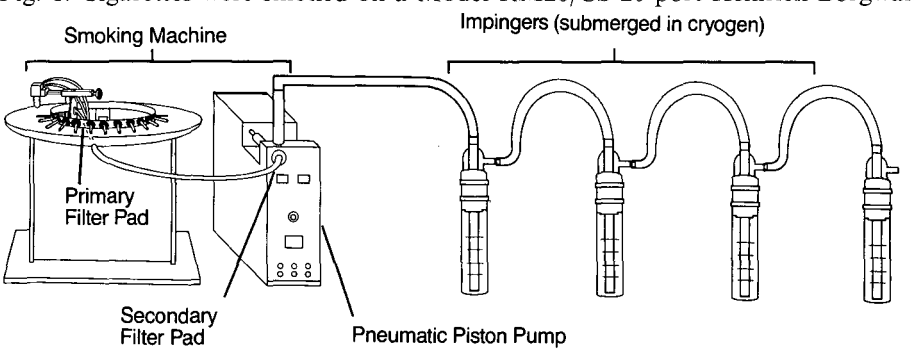



Fig. 1. Apparatus used for the collection of mainstream vapor phase cigarette smoke.

rotary smoking machine (Heinrich Borgwaldt, Hamburg, F.R.G.). The mainstream smoke was passed through a central Cambridge filter pad to remove particulate phase matter and through a secondary filter pad to ensure that no breakthrough of the particulate phase occured. All connections between the filter pads, the pneumatic piston pump, and the impingers were made with 0.25-inch Tygon tubings which had been previously rinsed with methanol. The effluent from the pneumatic piston pump was passed through four Midget Impingers (Ace Glass, Vineland, NJ, U.S.A.) connected in series. The impingers were modified to eliminate the constricted opening of the inlet tube and to extend the inlet tube length to within 1 mm of the bottom of the container. A 5-ml volume of methanol was placed in each impinger along with approximately 5 g of 3-mm glass beads in order to raise the level of methanol and to increase the cold surface area. The impinger joints were wrapped with Parafilm™ to effect an airtight seal. Each impinger was submerged in an isopropanol–dry ice cryogenic bath (−70°C).

*Smoking procedure and sample collection*

All cigarettes were smoked on the apparatus described above according to the Federal Trade Commission (FTC) puffing regimen (one 35 ml puff of 2 s duration every 60 s). The cigarettes were lit with a hydrogen flame. The 1R4F and the ultra-low-tar cigarettes were smoked to a butt length of 3 mm from the filter overwrap. Cigarettes B and C were smoked until the heat source was completely consumed. The butt length does not change during smoking of these cigarettes, and 9–10 puffs is their standard FTC smoking activity. Smoke was collected from 80 cigarettes for each sample except for the 1R4F cigarettes. Twenty 1R4F cigarettes were smoked per sample because of the relatively high analyte concentrations in the 1R4F mainstream vapor phase. For 1R4F cigarettes six samples were collected and analyzed. Three samples were collected and analyzed for cigarettes A, B and C.

When the smoking process described above was completed, 50 μl of the internal standard spiking solution were immediately added to each impinger. The impinger was capped with a solid stopper and vigorously shaken for 1 min with occasional venting. Samples of the impinger contents were transferred to GC vials and sealed with crimp caps.

Sample blanks were collected by inserting a cigarette filter into the smoking machine and dry puffing for an equivalent of 80 cigarettes.

*GC–MS analysis*

The GC–MS system used was a Hewlett-Packard HP 5970B MSD (Hewlett-Packard, Palo Alto, CA, U.S.A.) coupled to an HP 5890 GC via an open-split interface. The mass spectrometer was tuned by using perfluorotributylamine prior to analyzing a series of samples (every 2–3 days). An HP 7673 automatic liquid sampler was used to inject 1 μl of sample in the splitless mode (splitless time = 0.5 min). Analytes were separated on a J&W DB1-60W, 5.0-μm film, fused-silica capillary column (J&W Scientific, Folsom, CA, U.S.A.) by using helium as carrier gas at a head pressure of 22.5 p.s.i.g. The temperatures for the injection port and transfer line were 220°C and 250°C, respectively. For each analysis the GC oven was held at 35°C for 10 min. and then heated at 3°C/min to a temperature of 166°C. After elution of the analytes of interest, the column was heated at a rate of 50°C/min to 230°C and held for 5 min to clear the column of late eluting material.

The mass spectrometer was operated in the selected-ion monitoring mode. A separate chromatographic time window was used to monitor each analyte and its internal standard. Three ions each were monitored for the analyte and the internal standard (Table I). The scan frequency was 2.0 Hz. Each analyte and labeled internal standard was identified by retention time and the relative concordant responses of the multiple ions monitored. Only the molecular ions of each analyte and its internal standard were used for quantitation.

TABLE I

IONS MONITORED FOR EACH ANALYTE AND INTERNAL STANDARD

Ions shown in bold were used for quantitation.

| *Compound* | *Internal standard* | *Ions monitored* |
|---|---|---|
| Acrylonitrile | | 26, 52, **53** |
| | [$^2H_3$]Acrylonitrile | 26, 54, **56** |
| Benzene | | 39, 52, **78** |
| | [$^2H_6$]Benzene | 42, 56, **84** |
| Toluene | | 39, 78, **92** |
| | [$^2H_8$]Toluene | 42, 84, **100** |
| Styrene | | 39, 78, **104** |
| | [$^2H_8$]Styrene | 42, 84, **112** |

Response factors were determined daily by analyzing the series of four standard solutions. Quantitation was performed for each trap by using the method of internal standards. The total amount of the analyte per sample collection was obtained by summing the averages of duplicate injections across all four traps.

## RESULTS AND DISCUSSION

The method was assessed with regard to trapping efficiency, instrumental precision, and chromatography.

### *Trapping efficiency*

Fig. 2 is a plot of the amount of analyte per trap relative to the total amount in all the traps for the 1R4F and cigarette B. More than 75% of each compound was found in the first 1R4F trap and less than 1% was found in the final trap. With cigarette B, a greater percentage (more than 85%) was observed in the first trap presumably because less material was produced from this cigarette even though four times as many cigarettes were smoked as for the 1R4F. The last two traps show no analytes present for cigarette B. The diminishing amount in sequential traps for both cigarettes demonstrates excellent collection of the analytes in this study.

### *Instrument precision and linearity*

Instrumental precision was assessed by replicate injections of both a low-con-

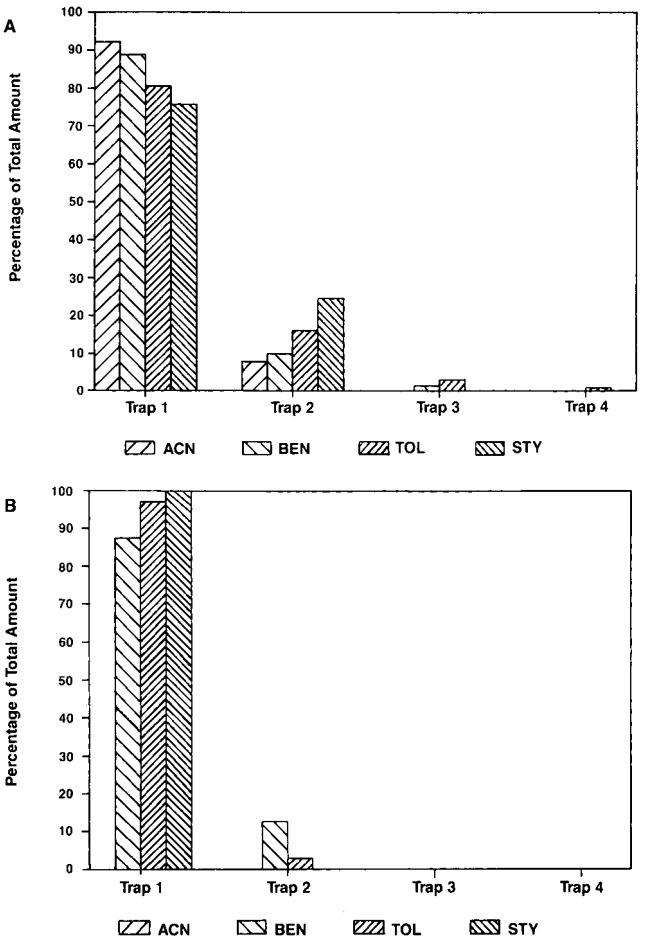



Fig. 2. Trapping efficiency plots for (A) 1R4F and (B) cigarette B samples. ACN = Acetonitrile; BEN = benzene; TOL = toluene; STY = styrene.

centration standard solution and a trap 1 sample from a ultra-low-tar cigarette. Average area response ratios and relative standard deviations (R.S.D. values) observed for each compound are summarized in Table II. Instrumental precision is comparable when estimated with either the standard solution or the smoke sample. R.S.D. values of 1.2% or less are observed for all compounds in the former case and R.S.D. values of 2% or less are observed for the latter. Instrument response linearity was determined from calibration plots of the standard solutions. In all cases, the $r^2$ value was greater than 0.99 for each analyte. The average R.S.D. values of the predicted values at their means were 2.4, 6.2, 8.2 and 1.4% for acrylonitrile, benzene, toluene and styrene, respectively[14].

TABLE II

AVERAGE AREA RESPONSE RATIOS OF UNLABELLED TO LABELLED $M^{+\cdot}$ FOR FIVE REPEATED INJECTIONS OF A LOW-CONCENTRATION STANDARD SOLUTION AND TRAP 1 OF AN ULTRA-LOW-TAR CIGARETTE

S.D. is the standard deviation and R.S.D. is the relative standard deviation.

| *Compound* | *Standard solution* | | *Ultra-low-tar cigarette* | |
|---|---|---|---|---|
| | *Average ± S.D.* | *R.S.D. (%)* | *Average ± S.D.* | *R.S.D. (%)* |
| Acrylonitrile/ [$^2H_3$]acrylonitrile | 0.411 ± 0.004 | 0.9 | 1.79 ± 0.04 | 2.1 |
| Benzene/ [$^2H_6$]benzene | 0.335 ± 0.001 | 0.2 | 4.38 ± 0.01 | 0.3 |
| Toluene/ [$^2H_8$]toluene | 0.230 ± 0.001 | 0.2 | 3.50 ± 0.01 | 0.3 |
| Styrene [$^2H_8$]styrene | 0.298 ± 0.004 | 1.2 | 1.61 ± 0.01 | 0.7 |

*Chromatography*

The non-polar column used in this work provided good resolution of all analytes. Typical chromatograms for acrylonitrile and a representative aromatic compound (styrene) are shown in Fig. 3 and 4. The acrylonitrile peak shape was broader and exhibited increased tailing compared to those of the aromatic compounds. As such, the method sensitivity and presicion for acrylonitrile were reduced relative to the other compounds studied. The limit of quantitation for acrylonitrile, defined as the concentration of acrylonitrile in the lowest response factor standard analyzed, was 0.2 μg/cigarette. As can be seen in Fig. 3B, the trace for *m/z* 53 shows a slight positive response for cigarette B. However, this response was below the limit of quantitation and too weak to determine if this response was due to acrylonitrile or an interference. All other compounds exhibited sharp, well-defined peaks with no observable background interference. The limits of quantitation for the aromatic compounds were 0.05 μg/cigarette.

*Quantitative data*

The measured amounts of each analyte and wet total particulate material (amount of material retained on the Cambridge filter pad) for each cigarette are summarized in Table III. R.S.D. values ranged from 3 to 27% with most being less than 15%. These results indicate that the sampling variability, which includes inherent cigarette variability and smoke collection variability, is greater than the instrumental variability described above. The amount of acrylonitrile determined in 1R4F smoke (7.6 μg/cigarette) falls in the range of reported values (3.2–15 μg/cigarette)[13]. The aromatic compound concentrations found in the 1R4F smoke also agree well with reported values (Table IV). Determination of benzene and toluene in mainstream vapor phase smoke by the procedure of Brunnemann *et al.*[17] for cigarettes that heat but do not burn tobacco shows fair agreement with our results for toluene

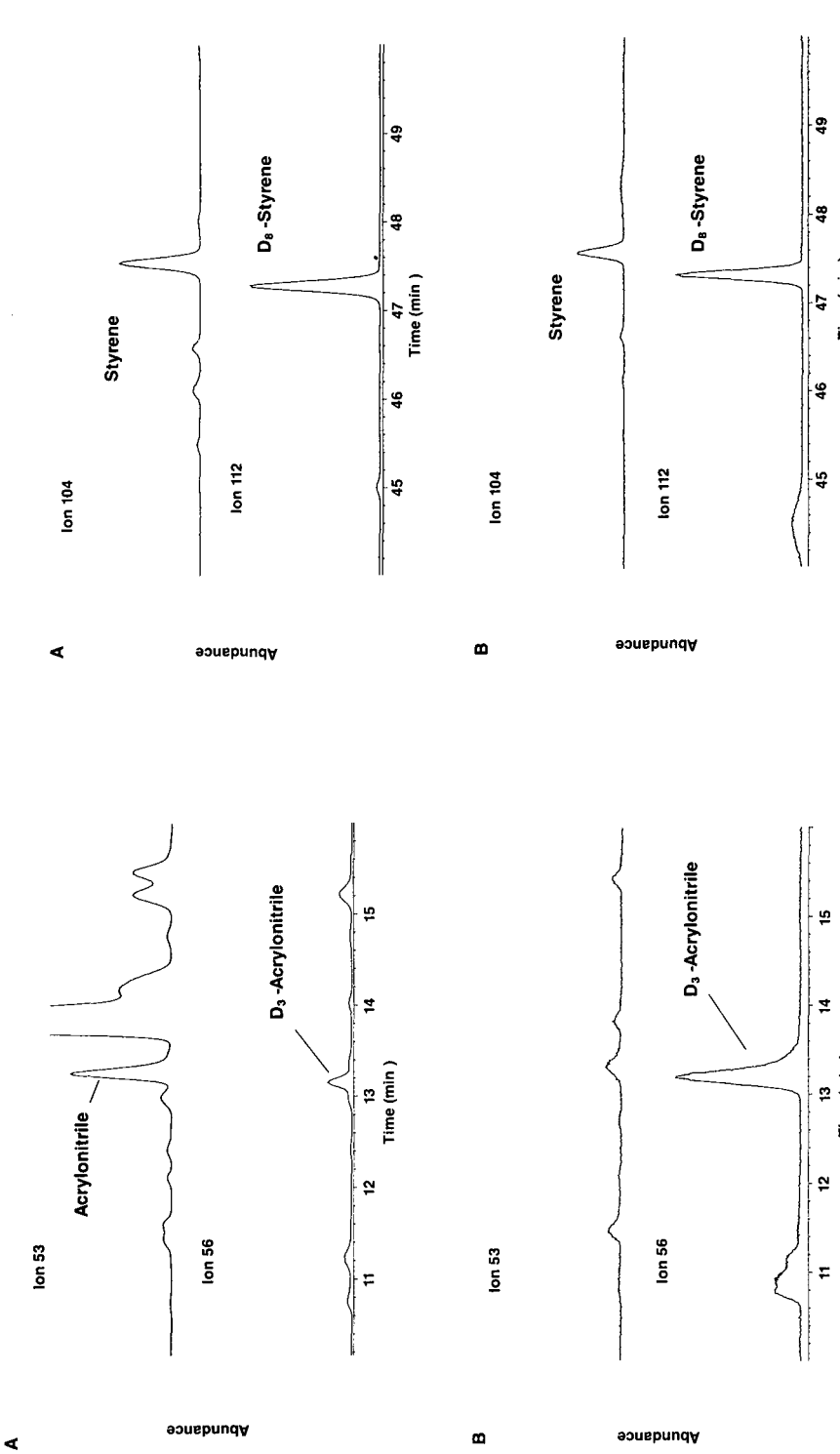



Fig. 3. Representative selected ion chromatograms for acrylonitrile and [$^2H_3$]acrylonitrile from trap 1 of samples (A) 1R4F and (B) cigarette B. $D_3$-Acrylonitrile = [$^2H_3$]Acrylonitrile.

Fig. 4. Representative selected-ion chromatograms for styrene and [$^2H_8$]-styrene from trap 1 of samples (A) 1R4F (B) cigarette B. $D_8$-Styrene = [$^2H_8$]Styrene.

TABLE III

AMOUNTS OF ANALYTES DETERMINED IN MAINSTREAM VAPOR PHASE SMOKE

| *Cigarette*[a] | *WTPM*[b] *(mg/cigarette)* | *Analyte (μg/cigarette)*[c,d] | | | |
|---|---|---|---|---|---|
| | | *Acrylonitrile* | *Benzene* | *Toluene* | *Styrene* |
| 1R4F | 11.4 | 7.6 ± 0.8 | 45 ± 3 | 68 ± 4 | 2.1 ± 0.3 |
| A | 1.5 | 0.6 ± 0.1 | 6.7 ± 0.2 | 5.9 ± 0.6 | 0.11 ± 0.03 |
| B | 9.0 | <0.2 | 1.9 ± 0.1 | 0.46 ± 0.04 | 0.077 ± 0.007 |
| C | 9.4 | <0.2 | 2.1 ± 0.1 | 0.7 ± 0.1 | 0.051 ± 0.008 |

[a]Cigarette A is a commercial ultra-low-tar mentholated brand; B is a cigarette that heats rather than burns tobacco; C is the mentholated version of B.

[b]Wet total particulate matter.

[c]The '±' number represents one standard deviation.

[d]Blank determinations yielded no measurable amount of any analyte.

(0.4 μg/cigarette) but a much lower value for benzene (0.3 μg/cigarette). The cause of this discrepancy may be variations in the lighting technique for these types of cigarettes.

Of the cigarettes studied, the 1R4F smoke contained the greatest concentrations of each analyte. Cigarette A has a wet total particulate matter (WTPM) that is 87% less than that of the 1R4F and, correspondingly, all of the analytes in A are reduced relative to the 1R4F by 85–95%. However, the WTPM values for cigarettes B and C (those that heat rather than burn tobacco) are similar to that of the 1R4F but still show more than 90% reduction of the analytes relative to 1R4F. Even if acrylonitrile had been detected in cigarettes B and C at its quantitation limit of 0.2 μg/cigarette, a reduction of 97% relative to 1R4F would have been observed. The analyte reductions for cigarettes B and C relative to both the 1R4F and the ultra-low-tar

TABLE IV

COMPARISON OF RESULTS FOR AROMATIC COMPOUNDS IN THE 1R4F WITH OTHER REPORTED VALUES

| *Compound* | *μg/cigarette* | | |
|---|---|---|---|
| | *This work* | *Brunnemann et al.*[a] | *Higgins et al.*[b] |
| Benzene | 45 | 51 | 42 |
| Toluene | 68 | 73 | 55 |
| Styrene | 2.1 | – | 3 |

[a]Values for 1R4F[7]. The 1R4F yields 9.2 mg tar under FTC conditions.

[b]Values for a filtered American commercial brand with a 7 mg tar yield[8].

cigarette A suggest a simpler smoke chemistry for these cigarettes. This is not surprising since heating instead of burning tobacco would be expected to yield a less complex smoke. The visual appearance of the samples also supports this premise. Trap 1 samples from the Kentucky reference 1R4F and the ultra-low-tar cigarette A were slightly discolored while comparable samples from cigarettes B and C were virtually colorless.

## REFERENCES

1 M. F. Dube and C. R. Green, *Recent Adv. Tobacco Sci.*, 8 (1982) 42.
2 *New Cigarette Prototypes that Heat Instead of Burn Tobacco*, R. J. Reynolds Tobacco Co., Winston-Salem, NC, 1988.
3 R. M. Irby, Jr. and E. S. Harlow, *Tobacco Sci.*, 3 (1959) 52.
4 A. D. Horton and M. R. Guerin, *Tobacco Sci.*, 18 (1974) 19.
5 H. Elmenhorst and R. Tschesche, *Beitr. Tabakforsch.*, 3 (1965) 101.
6 M. S. Baggett, G. P. Morie, M. W. Simmons and J. S. Lewis, *J. Chromatogr.*, 97 (1974) 79.
7 K. D. Brunnemann, M. R. Kagan and D. Hoffmann, *42nd Tobacco Chemists' Research Conference, Lexington, Kentucky, October 2–5, 1988.*
8 C. E. Higgins, W. H. Griest and G. Olerich, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 1074.
9 A. J. Nunez, L. F. Gonzalez and J. Janak, *J. Chromatogr.*, 300 (1984) 127.
10 G. MacLeod and J. M. Ames, *J. Chromatogr.*, 355 (1986) 393.
11 B. N. Colby, P. W. Ryan and J. E. Wilkinson, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 72.
12 *Smoking and Health, a Report of the Surgeon General*, U.S. Dept. Health, Education and Welfare, DHEW Publication No. (PHS) 79-50066, Washington, DC, 1979.
13 *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 38, Tobacco Smoking*, World Health Organization, Geneva, 1985.
14 J. C. Miller and J. N. Miller, *Statistics for Analytical Chemistry*, Ellis Horwood, Chichester, 2nd ed., 1988, Ch. 5.

*Journal of Chromatography*, 503 (1990) 369–375
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 169

# Determination of primary and secondary aliphatic amines in the environment as sulphonamide derivatives by gas chromatography–mass spectrometry

AKIKO TERASHI*, YOSHIFUMI HANADA, AZUMA KIDO and RYOTA SHINOHARA
*Kitakyusyu Municipal Institute of Environmental Health Sciences, 1-2-1, Shin-ike, Tobata-ku, Kitakyusyu-shi, Fukuoka 804 (Japan)*
(First received August 2nd, 1989; revised manuscript received November 13th, 1989)

SUMMARY

A gas chromatographic–mass spectrometric (GC–MS) method for the determination of primary and secondary aliphatic amines in water and sediment was developed. A standard solution of amines was added to river water, sea water and sea sediment, and distilled under alkaline conditions. The distillate was reacted with benzenesulphonyl chloride to form the corresponding sulphonamides. After extracting the derivatives into dichloromethane, the organic layer was concentrated to a definite volume. The determination was carried out by GC–MS with selected-ion monitoring. The detection limits of amines in water and sediment were 0.02–2 μg/l and 0.5–50 μg/kg, respectively. The recoveries were 68.4–98.8%.

## INTRODUCTION

Aliphatic amines are broadly distributed in the environment, reflecting the fact that they are produced in living bodies as metabolites. Amine compounds have also been widely used as raw materials for pesticides, medicines, dyestuffs, etc. Not only are some amines themselves hazardous to human health, but some may form nitrosamines, known to be carcinogenic by reaction with nitrates[1–3]. It is therefore important to determine the concentration levels of amines in the environment.

Amines are usually determined by gas chromatography (GC) or high-performance liquid chromatography (HPLC), but methods have some problems[4]. Handling of low-molecular-weight amines is difficult because of their high water solubility and volatility. Moreover, in GC analysis, they are likely to be adsorbed and decompose in the column, and readily give tailed elution peaks. LC analyses also have the problem of low detection sensitivity and selectivity on ultraviolet (UV) detection. Derivatizations are popular methods for overcoming these problems.

In recent years, there have been many reports concerning HPLC analyses of amines by using UV[5–7], fluorescence[8–12], chemiluminescence excitation[13] or electro-

0021-9673/90/$03.50 

chemical detection (ED)[14,15] with appropriate derivatization. Some of them are very sensitive, but it needs to be examined whether their selectivities are satisfactory for environmental samples with complicated matrices.

Many derivatization reagents for GC analyses of amines by using electron-capture detection (ECD)[16–18], flame thermionic detection (FTD)[19], flame photometric detection (FPD)[20,21] or GC–mass spectrometry with selected-ion monitoring (GS–MS–SIM)[22] have also been reported. Benzenesulphonyl chloride (BSC)[19,20] and 2,4-dinitrofluorobenzene (DNFB)[4,23] have proved to be very useful for low-molecular-weight aliphatic amines because they can convert the amines into hydrophobic and non-volatile derivatives in water. The reaction procedures are simple, but DNFB irritates the skin and occasionally cause allergic dermatoses.

In this study, BSC was used as a derivatization reagent and the GC–MS–SIM determination of some aliphatic primary and secondary amines in environmental samples was examined.

## EXPERIMENTAL

### *Chemicals*

*n*-Propylamine (nPA), isopropylamine (iPA), n-butylamine (nBA), allylamine (AA), *n*-octylamine (nOA), di-*n*-propylamine (DPA), diisobutylamine (DBA), di(2-ethylhexyl)amine (DEHA), [$^2H_{10}$]fluoranthene [as an internal standard (IS)] and BSC were purchased at the highest purity available and used without further purification. Dichloromethane and hexane were of pesticide grade and all other reagents were of analytical-reagent grade.

### *Apparatus*

A Model DX303 mass spectrometer (JEOL) coupled with a Model 5890A gas chromatograph (Hewlett-Packard) was used under the following conditions: column, 25 m × 0.53 mm I.D., SE-54; column temperature, 200°C (1 min), increased at 10°C/min to 280°C, held for 3 min; injection temperature, 280°C; carrier gas, helium at 13.0 ml/min; GC–MS interface temperature, 280°C; ionization voltage, 70 eV; and sample size, 2 μl of diluted samples.

For GC–FID, A Model JGC-20K gas chromatograph (JEOL) equipped with a 1-m 3% OV-17–1% PZ179 packed column and a Model G-3000 gas chromatograph (Hitachi) equipped with a 25 m × 0.53 mm I.D. SE-54 column were used for preliminary examinations to determine the optimum conditions.

### *Procedure*

A water sample (500 ml) was placed in a round-bottomed flask, adjusted to pH 10 with 0.2 *M* sodium hydroxide solution and distilled. The distillate (100 ml) was collected in a separating funnel. The condenser was washed with 0.1 *M* hydrochloric acid. To the distillate, 3 g of sodium hydroxide and 7 ml of BSC were added and the mixture was shaken vigorously for 30 min, then a further 3 g of sodium hydroxide were added and the mixture was shaken for 10 min to decompse the excess of BSC. This solution was adjusted to pH 5 with 50% hydrochloric acid and extracted twice with 10 ml of dichloromethane. The organic layer was separated and concentrated to 1 ml with a Kudema–Danish evaporator. After 10 μl of 10 ppm [$^2H_{10}$]fluoranthene

(I.S.) solution had been added, 2 $\mu$l were injected into the GC system. A series of standard solutions were treated using the same procedure, except for the distillation process, and calibration graphs were constructed by comparing their peak heights with that of the internal standard.

If an interference was observed on the GC–MS-SIM trace, the sample solution was treated as follows. Hexane was added to the sample solution to a total volume of *ca.* 2 ml. A silica gel column (10 cm × 10 mm I.D.) was prepared by washing with hexane, then the sample solution was placed on it. The column was washed with 20 ml of hexane–dichloromethane, then the amines were eluted with 30 ml of dichloromethane. The eluate was concentrated to 1 ml with a Kuderna–Danish evaporator.

## RESULT AND DISCUSSION

### *Reaction conditions*

The reaction yield depends on the amounts of BSC and sodium hydroxide. When 7 ml of BSC (0.055 mol) and 3 g of sodium hydroxide (0.075 mol) were added, the rate was found to be optimum.

### *Distillation conditions*

The recovery of the distillation procedure was maximum at pH 10. Sodium chloride as a salting-out reagent had a negative effect on the recovery efficiency.

Individual amine standards (1000 $\mu$g) were added to 500 ml of water and distilled at pH 10. The distillate was fractionated every 10 ml, and each fraction was treated in the same way as above. The washing solution from the condenser was combined with the last fraction (90–100 ml). The amine concentrations were determined by GC–FID.

The distillation curves are shown in Fig. 1. All amines were eluted within 100

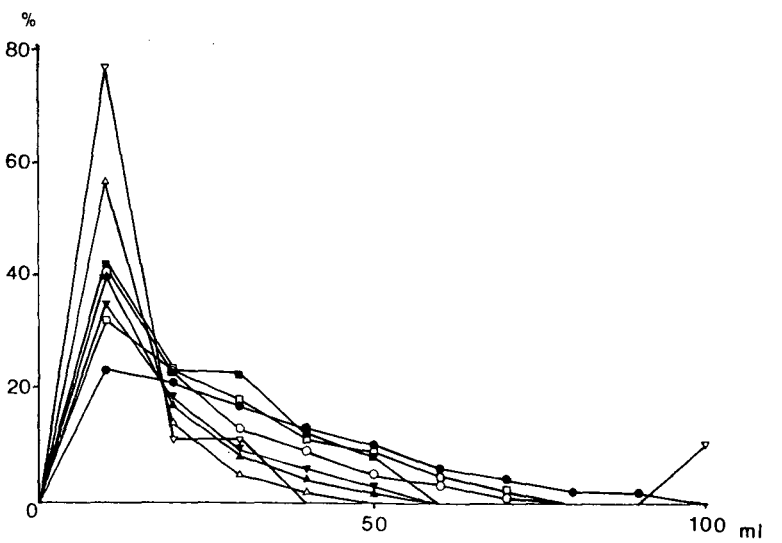



Fig. 1. Distillation curves for aliphatic amines. ○ = iPA; ● = AA; □ = nPA; ■ = nBA; △ = DPA; ▲ = DBA; ▽ = nOA; ▼ = DEHA.

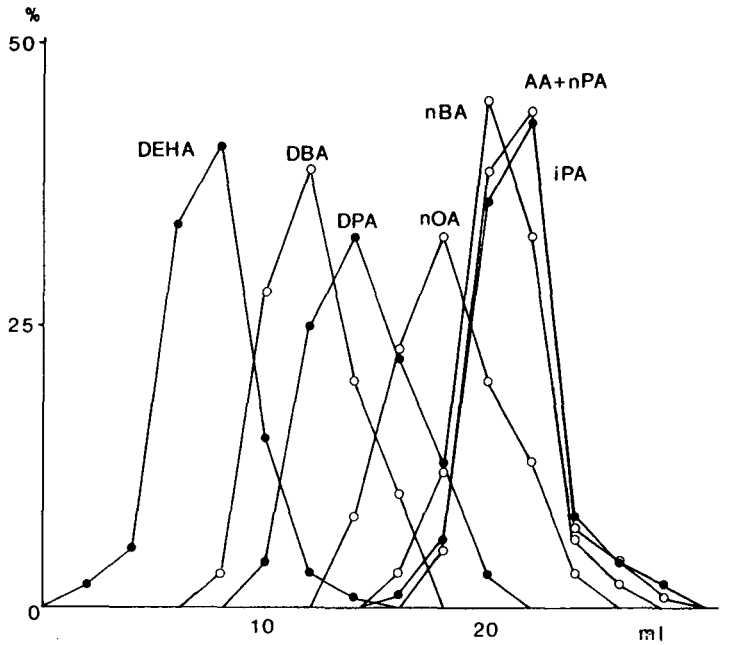



Fig. 2. Elution curves for amine–BSC derivatives obtained by silica gel column chromatography.

ml. Detection of *n*-octylamine in the last fraction can be attributed to the 0.1 *M*-hydrochloric acid washing of the condenser. n-Octylamine seems to be easily adsorbed on the glass wall and may fail to be recovered without washing.

*Silica gel column chromatography*

Clean-up was examined with a silica gel column because environmental samples usually contain many interfering substances. A standard mixture in dichloromethane

TABLE I

DETECTION LIMITS OF AMINES FOUND BY GC–MS–SIM

Water sample, 500 ml; sediment sample, 10 g (dry base).

| *Compound* | *m/z* | *Detection limit* | |
|---|---|---|---|
| | | *Water (μg/l)* | *Sediment (μg/kg)* |
| iPA | 184 | 0.06 | 3 |
| AA | 170 | 2 | 100 |
| nPA | 170 | 0.07 | 3.5 |
| nBA | 170 | 0.06 | 3 |
| DPA | 212 | 0.01 | 0.5 |
| DBA | 226 | 0.01 | 0.5 |
| nOA | 170 | 0.05 | 2.5 |
| DEHA | 184 | 0.03 | 1.5 |
| I.S. | 212.1 | – | – |

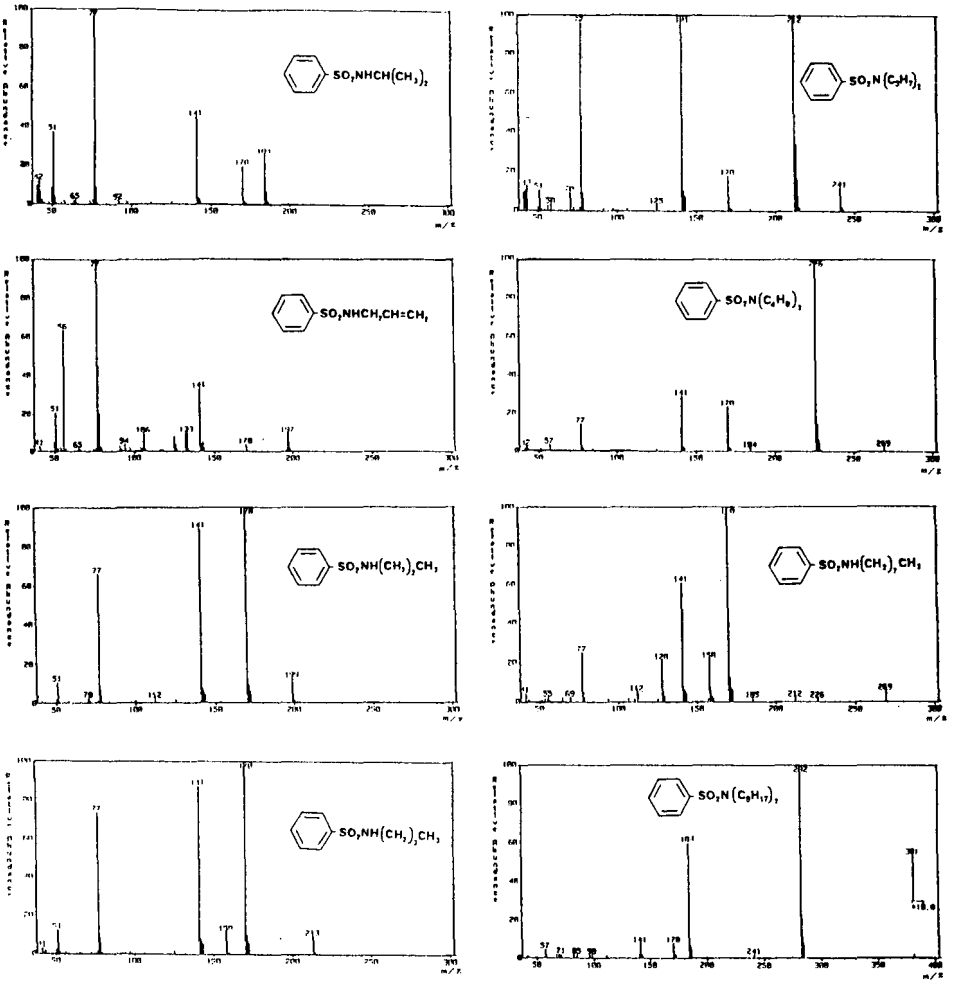



Fig. 3. Mass spectra of amine–BSC derivatives.

containing 100 ppm of each amine–BSC derivative was prepared. This solution (5 ml) was concentrated to 1 ml and diluted with same volume of hexane. Silica gel column chromatography was then carried out by the procedure mentioned above. The eluate was fractionated every 2 ml and the concentration of each amine in each fraction was determined by GC–FID. Fig. 2 shows the elution curves. No amines were eluted with hexane–dichloromethane (1:1), and all of them were completely eluted with 30 ml of dichloromethane.

*Derivatization*

The reaction of amines and BSC is

$$R_1R_2NH + C_6H_5SO_2Cl \rightarrow C_6H_5SO_2NR_1R_2 + HCl$$

TABLE II

RECOVERIES OF AMINES FROM ENVIRONMENTAL SAMPLES ($n=4$)

R.S.D. = Relative standard deviation.

| *Compound* | *River water* | | *Sea water* | | *Sea sediment* | |
|---|---|---|---|---|---|---|
| | *Recovery (%)* | *R.S.D. (%)* | *Recovery (%)* | *R.S.D. (%)* | *Recovery (%)* | *R.S.D. (%)* |
| iPA | 98.8 | 4.5 | 92.3 | 3.8 | 89.7 | 5.2 |
| AA | 90.2 | 2.4 | 87.2 | 3.6 | 68.4 | 3.8 |
| nPA | 87.6 | 4.3 | 84.9 | 7.2 | 85.5 | 3.8 |
| nBA | 97.2 | 5.1 | 73.0 | 4.6 | 68.5 | 3.6 |
| DPA | 92.4 | 3.3 | 98.0 | 5.1 | 69.0 | 1.0 |
| DBA | 90.8 | 5.6 | 92.1 | 3.4 | 96.2 | 5.3 |
| nOA | 94.2 | 3.9 | 88.9 | 9.1 | 73.7 | 2.2 |
| DEHA | 91.9 | 3.4 | 88.3 | 4.5 | 72.3 | 9.3 |

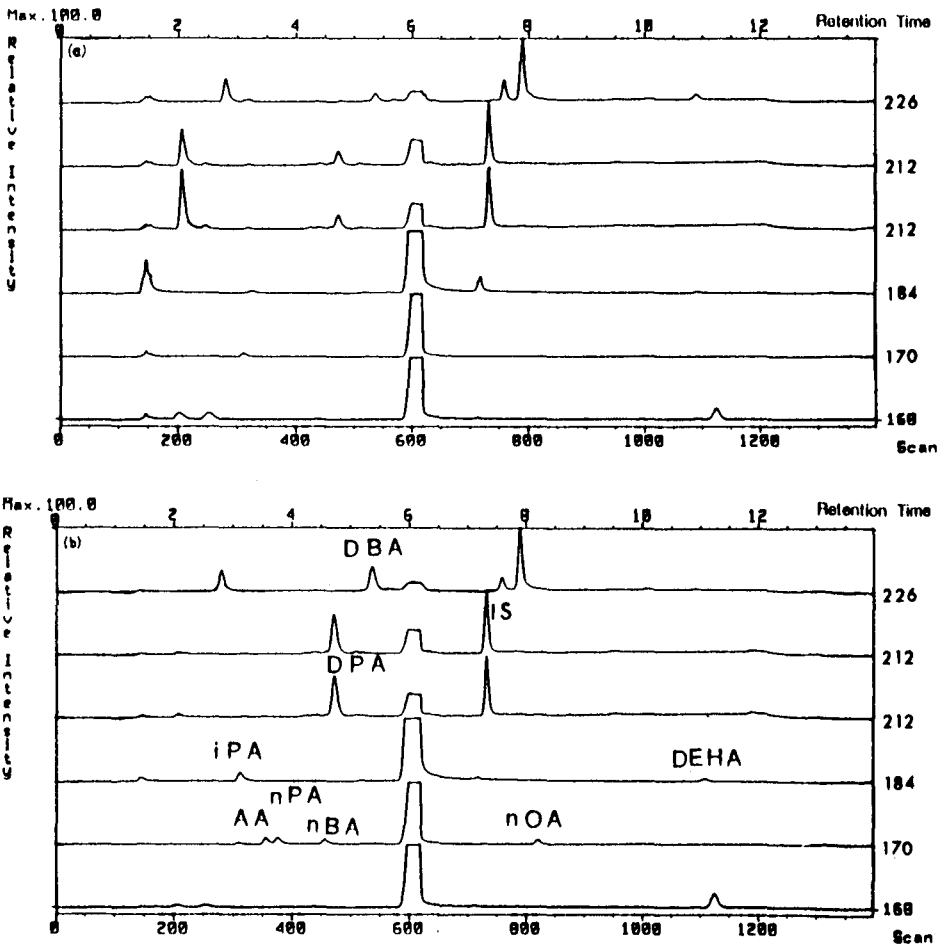



Fig. 4. SIM chromatograms of amine–BSC derivatives in a sea-water sample: (a) unspiked; (b) spiked.

where R = alkyl or H. This reaction can be performed in an early step in the analysis in water. It is reasonable to derivatize the amines prior to the concentration procedure in order to prevent the loss of some low-molecular-weight amines that are liable to volatilize.

Mass spectra of amine–BSC derivatives are shown in Fig. 3. From these mass spectra, suitable fragment ions were chosen for GC–MS–SIM.

The detection limits of the amines are shown in Table I (signal-to-noise ratio = 3). Allylamine has a relatively high detection limit because it has few intensive fragment ions (see Fig. 3).

Recovery tests were carried out by using river water, sea water and sea sediment spiked with amounts of each amine representing about ten times the detection limits. The results are given in Table II and representative chromatograms are shown in Fig. 4. nBA, DPA and DBA were detected in trace amounts in unspiked samples.

## REFERENCES


1 E. A. Walker, P. Bogovski and L. Griciute, *Environmental N-Nitroso Compounds, Analysis and Formation (IARC Scientific Publication*, No. 14), International Agency for Research on Cancer, Lyon, 1976.
2 M. C. Bowman, *Handbook of Carcinogens and Hazardous Substances*, Marcel Dekker, New York, Basle, 1982.
3 R. A. Scanlan and S. R. Tannenbaum, *N-Nitroso Compounds*, American Chemical Society, Washington, DC, 1981.
4 D. R. Knapp, *Handbook of Analytical Derivatization Reactions*, Wiley, New York, 1979.
5 F. V. Carlucci and E. Karmas, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 564–568.
6 E. S. Barreira, J. P. Parente and J. W. Alencar, *J. Chromatogr.*, 398 (1987) 381–386.
7 K. Andersson, C. Hallgren, J. L. Levin and C. A. Nilsson, *J. Chromatogr.*, 312 (1984) 482–488.
8 S. C. Beale, J. C. Savage, D. W. Shawn, M. Wietstock and M. Novotny, *Anal. Chem.*, 60 (1988) 1765–1769.
9 P. Lindroth and K. Mopper, *Anal. Chem.*, 51 (1979) 1667–1674.
10 K. Samejima, *J. Chromatogr.*, 96 (1974) 250–254.
11 R. H. Buck and K. Krummen, *J. Chromatogr.*, 303 (1984) 238–243.
12 P. Jandera, H. Pechova, D. Tocksteinova, J. Churacek and J. Kralovsky, *Chromatographia*, 16 (1982) 275–281.
13 G. Melbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203–210.
14 T. Chou, S. T. Colgan, D. M. Kao and I. S. Krull, *J. Chromatogr.*, 367 (1986) 335–344.
15 W. A. Jacobs, *J. Chromatogr.*, 392 (1987) 435–441.
16 A. C. Moffat, E. C. Horning, S. B. Matin and M. Rowland, *J. Chromatogr.*, 66 (1972) 255–260.
17 A. J. Francis, E. D. Morgan and C. F. Poole, *J. Chromatogr.*, 161 (1978) 111–117.
18 F. E. Scully, Jr., R. D. Howell, H. H. Renn and K. Mazina, *Environ. Sci. Technol.*, 22 (1988) 1186–1190.
19 K. Jacob, C. Falkner and W. Vogt, *J. Chromatogr.*, 167 (1978) 67–75.
20 T. Hamano, Y. Mitsuhasi and Y. Matsuki, *J. Chromatogr.*, 190 (1980) 462–465.
21 T. Hamano, A. Hasegawa, K. Tanaka and Y. Matsuki, *J. Chromatogr.*, 179 (1979) 346–350.
22 K. Jacob, W. Voty, C. Krauss, G. Schnabl and M. Knedel, *Biomed. Mass Spectrom.*, 10 (1983) 175–182.
23 M. Koga, T. Akiyama and R. Shinohara, *Bunseki Kagaku*, 30 (1981) 745–750.

*Journal of Chromatography*, 503 (1990) 377–383
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 191

# Determination of chlorpyrifos in water by large-volume direct aqueous injection capillary gas chromatography

BRUCE B. GERHART and HERNAN J. CORTES*
*The Dow Chemical Company, Analytical Sciences, 1897 Building, Midland, MI 48677 (U.S.A.)*
(First received September 5th, 1989; revised manuscript received December 5th, 1989)

## SUMMARY

A technique is described for the injection of large-volume aqueous samples in capillary gas chromatography for the direct determination of chlorpyrifos in well water. An uncoated inlet of suitable length coupled to a thin-film methyl phenyl silicone capillary column was employed for the determination of low parts-per-$10^9$ (ppb) levels of the pesticide in water. Samples were injected without prior clean-up steps by using a 10-port valve, and detection was performed by electron capture. The method yielded high accuracy, precision (4.8% with 95% confidence at 0.9 ppb), and suitable linearity range (0.9–18 ppb). The method presented circumvents the extraction, filtration and centrifugation steps commonly used in the determination of trace organic components in aqueous matrices.

## INTRODUCTION

The emphasis on the analysis of trace organic compounds in aqueous samples has increased in the past two decades[1]. A major problem in this area has been the gas chromatographic quantitation of organic pollutants at trace levels by the direct injection of aqueous samples[2–6]. Pesticide analysis in particular has usually been performed by extraction using an organic solvent and subsequent concentration prior to injection of small volumes into a gas chromatograph[7,8].

Recently, advancements have been recognized in the on-line coupling of reversed-phase liquid chromatography (LC) with capillary gas chromatography (GC) for the determination of organic trace constituents in complex matrices[9,10]. This paper presents a gas chromatographic system for the analysis of chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphothioate] by the direct injection of relatively large volumes of an aqueous sample with electron-capture detection. The analysis procedure reduces the number of off-line manipulation and concentration steps thereby providing simplicity and automation, and the possibility of higher sample throughput. By employing a non-deactivated fused-silica inlet (retention gap) coupled to a thin-film methylsilicone capillary column, chlorpyrifos was sufficiently resolved from inter-

0021-9673/90/$03.50 

ferences while maintaining peak shape integrity at the party-per-billion[a] (ppb) level. Though the "retention gap" technique is generally recognized as not being compatible with aqueous solvents[11], preliminary data obtained in our laboratories[12] indicated that relatively large volumes of water could be injected into a capillary GC column without detrimental effects on peak shape and resolution, and the present chromatographic set-up takes advantage of phase-ratio focusing[13] and cold-trapping mechanisms[14], while utilizing the non-deactivated fused-silica inlet to allow complete solvent vaporization prior to reaching the separation column.

## EXPERIMENTAL

A system diagram for the determination of chlorpyrifos in water is presented in Fig. 1. The gas chromatograph used was a Hewlett-Packard (Avondale, PA, U.S.A.) 5890. A 10-port valve (Model A4C10WT, Valco, Houston, TX, U.S.A.) was mounted 5 cm outside the oven wall and was equipped with a stainless-steel external sample loop of 20 $\mu$l volume. A 20 m × 0.25 mm non-deactivated fused-silica capillary (Polymicro Technologies, Pheonix, AZ, U.S.A.) used as an inlet was contained within the oven and was directly attached to the valve. This uncoated inlet was connected via a low dead volume union (ICT, Model IC25250) to a 30 m × 0.25 mm, 5% phenylmethylsilicone capillary of 0.25 $\mu$m film thickness (J&W Scientific, Folsom, CA, U.S.A.). An electron-capture detector operated at 350°C was used. Integration and recording of the signals was performed by a Hewlett-Packard 3392A integrator. Typical operating conditions were helium carrier at 6.4 ml/min (at 130°C) and oven temperature of 130°C for 15 min followed by a program of 20°C/min to 280°C. Make-up flow to the detector was argon–methane (10:90) at 32 ml/min.

A Hewlett-Packard 19405A sample/event control module was set up to control a Valco digital valve interface (DVI) and an a Hewlett-Packard 3392A integrator. The DVI drove a Valco helical-drive air actuator (AT104) which in turn rotated the 10-port

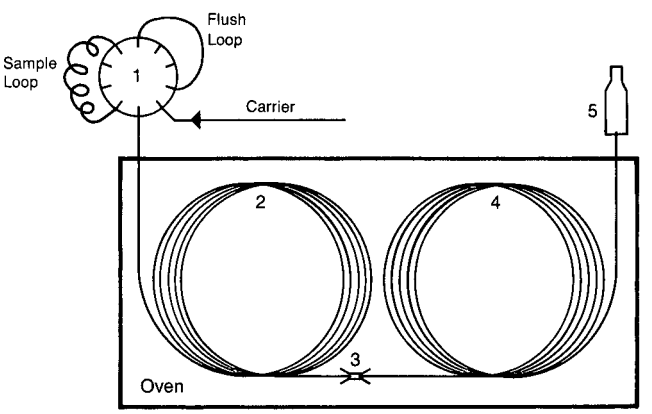



Fig. 1. Schematic diagram of the system for chlorpyrifos determination in water. 1 = Injection valve with sample loop (20 $\mu$l stainless steel) and flush loop; 2 = uncoated inlet; 3 = press-fit connector; 4 = capillary GC column; 5 = electron-capture detector.

[a] Throughout this paper, the American billion ($10^9$) and trillion ($10^{12}$) are meant.

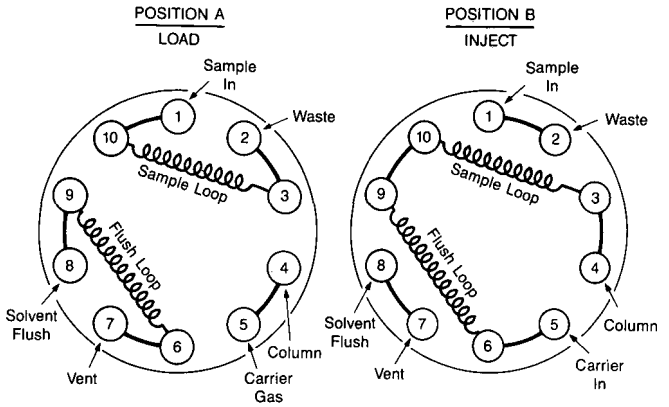



Fig. 2. 10-Port injection valve. Position A: loading of sample and solvent flush; position B: injection of sample onto the capillary GC column.

valve between load and inject positions. A Micromeritics 725 autoinjector was used to fill the sample loop of the valve and control the timing between each run. A schematic of the 10-port injection valve configuration is shown in Fig. 2. A flush loop was installed ahead of the sample loop on the valve to study the effects of flushing the aqueous sample with organic solvent in order to minimize possible carry-over.

## RESULTS AND DISCUSSION

To achieve sufficient resolution and undistorted peak shapes, the component of interest must be retained at the head of the capillary GC column while the injected water is eluted at or near its boiling point. Once the component of interest was reconcentrated, the oven temperature was increased to elute the higher boiling component. All of the water passed through the 5% phenylmethylsilicone capillary and the electron-capture detector. Collection and processing of the detector signal was started after all the water had passed through the detector. Several variables were examined in the development of the optimum chromatographic conditions, as a critical balance had to be made between injecting sufficient sample size to provide an adequate detection limit and the volume of uncoated inlet necessary to allow solvent evaporation without allowing liquid to reach the stationary phase of the capillary GC column. Carrier gas flow-rate was adjusted well above optimum (6.4 ml/min at 130°C) so as to rapidly move the sample out of the injection loop and through the tandem capillar system. Flow was mantained by a constant pressure regulator, thus the helium carrier decreased in flow-rate to 4.4 ml/min at 280°C.

Initially, injections were made by using a stainless-steel sample loop with the loop open to the carrier for the full length of the analysis. A long band of baseline noise appeared after the majority of water eluted, attributed to residual water adsorbed to the walls of the injection loop (Fig. 3A). This was also observed by using a non-deactivated section of fused silica as the injection loop, and has been previously reported[10]. Timed injections of 30–45 s eliminated the noise (due to slow desorption of water in the loop), without observed carry-over of sample or detrimental effects in

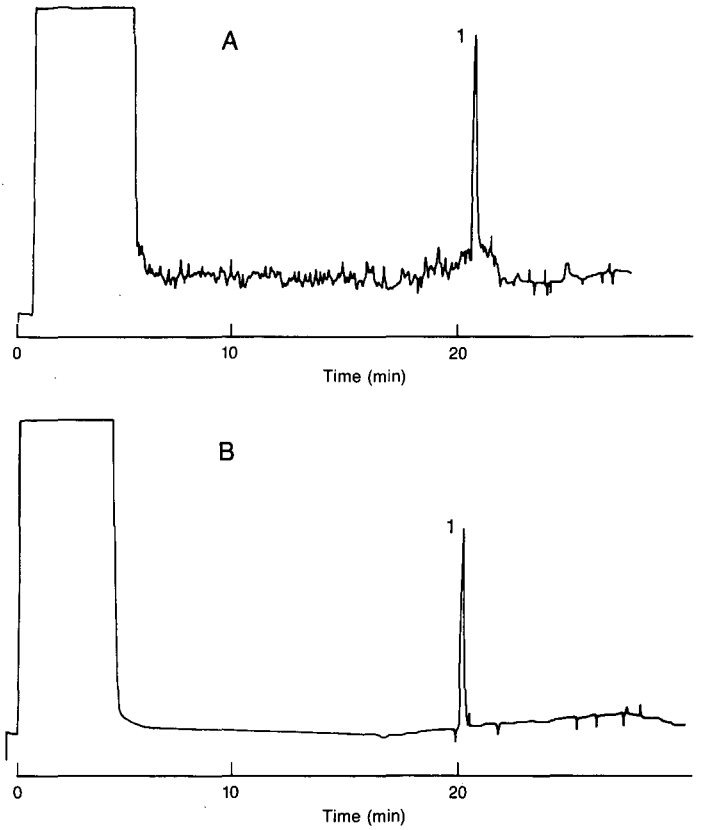



Fig. 3. Chromatograms of chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate] in water. Column: 20 m × 0.25 mm I.D. DB-5, 0.25-$\mu$m film. Uncoated inlet: 20 m × 0.25 mm I.D. non-deactivated fused silica. Oven temperature: 130°C for 15 min, program to 250°C at 10°C/min. Carrier gas: helium at 6.4 ml/min. Detector: electron-capture detector at 350°C. Make-up gas: argon–methane (10:90) at 32 ml/min. Injection size: 20 $\mu$l. (A) Valve in the inject position for the full length of the analysis. (B) Valve in the inject position for 45 s. Peak 1 = chlorpyrifos 12 ng/ml.

quantitation (Fig. 3B). By using the conditions listed, the use of a solvent in the flush loop was found to be unnecessary.

*Linear response for the electron-capture detector*

Linearity of the electron-capture detector response for chlorpyrifos in the water matrix was investigated by using two different make-up gases. A linear range of response with a correlation coefficient of 0.97 was obtained in the range of 0.9 to 18 ppb chlorpyrifos with either nitrogen or argon–methane (10:90) as the detector make-up gas. This is a range typically seen for this selective detector[15]. In spite of the limited linear range of the electron-capture detector, this procedure lends itself well to expanding the workable range of chlorpyrifos concentrations through appropriate dilution of the sample or decreased injection volumes.

*Precision of analysis*

The precision of the method was tested at a level of 0.9 ppb (ng/ml) chlorpyrifos dissolved in distilled water. Table I lists the results obtained for replicate determinations at the 0.9 ppb level. It was observed that the precision of the analysis could be greatly increased by determining peak height with forced baseline at the valley points (4.8 *vs.* 33% when using integrated areas for calculation). The detection limit of the reported procedure was calculated as 220 ppt (three times baseline random noise level).

TABLE I

RECOVERY OF 0.9 PARTS PER BILLION (ng/ml) CHLORPYRIFOS IN WATER

| | *Concentration found (ng/ml)* | |
|---|---|---|
| | *Integrated peak area* | *Peak height with forced baseline* |
| | 0.73 | 0.89 |
| | 0.87 | 0.88 |
| | 0.99 | 0.87 |
| | 1.02 | 0.89 |
| | 0.71 | 0.84 |
| Mean | 0.86 | 0.87 |
| S.D. | 0.14 | 0.02 |
| R.S.D. (%) | 16 | 2.3 |

*Column performance and stability*

Much of the hesitancy to perform aqueous GC on a routine basis is the suspected incompatability and deleterious effect that water has on the stationary phases used. The question in our research group concerned the possibility of phase alteration that might lead to increased adsorbtion and column activity toward the component of interest and/or non-reproducible retention times. As a result, detailed records were maintained of the number of injections, the volume of water, and the volume of organic wash solvents introduced to the system. A polar test mixture (CP-8) was chromatographed prior to the aqueous injections and, later, periodically to track the column integrity. Typical observations for the bonded 5% phenylmethylsilicone capillary columns were 150 aqueous injections of 20 $\mu$l each without discernable change in column activity or retention times. Columns that have had more than 3 ml of water passed through them at elevated column temperatures are still being used and do not appear to have experienced measurable degradation. It is concluded that the original column retention behavior is maintained for a sufficient number of aqueous injections to make the daily routine use of this method quite economical.

*Automation of the analytical instrument*

The chromatographic system lends itself well to computer-controlled automated analysis. A Micromeritics LC autosampler, a Valco DVI and a Valco helical-drive air

actuator (AT104) were connected to a Hewlett-Packard 19405A sample/event control module. Approximately 50 samples (calibration standards, water blanks, and organic wash solvents) can be analyzed along with the actual well water samples without manual intervention. It should be noted that carry-over can take place with samples of particularly high levels of chlorpyrifos (>100 ppb); however, the positioning of acetone or methanol in sample vials between each sample on the autosampler effectively eliminated detectable carry-over, thereby safeguarding all analytical runs. Samples with high chlorpyrifos levels could be diluted with water and reanalyzed.

*Well water sample analysis*

Test were conducted to determine whether direct aqueous injection would provide comparable results to the method typically used for this type of analysis. In this method, a 40-ml aliquot of the water sample is extracted with 2 ml of hexane. After shaking for 15 min and centrifuging for 3 min at 6 *g*, a 5-$\mu$l portion of the hexane layer is injected onto a 180 cm × 3 mm I.D. × 6.4 mm O.D. borosilicate glass column packed with a mixed phase of OV-17 and QF-1 with 11% loading on 80–100-mesh Gas-Chrom Q, at an oven temperature of 205°C. Carrier gas used was nitrogen at 20 ml/min and detection via a flame photometric detector. Calibration of the instrument was performed with a 1 ppb standard of chlorpyrifos in hexane.

In the direct aqueous injection procedure, external standard calibration was performed by forming a least squares plot of three standards containing between 0.9 and 18 ppb chlorpyrifos. The direct injection procedure indicated variability of ± 5% at levels of 30 ppb and ± 25% at 1 ppb compared to the values generated by existing methodology.

## CONCLUSIONS

Large-volume direct aqueous injection GC for the determination of chlorpyrifos in water shows advantages over existing methodology by providing rapid and reliable analyses of environmental water samples. Extraction steps are completely eliminated and it has been shown that the integrity of the capillary column can be maintained and that precision is adequate at quantitation levels of 0.9 to 18 ppb. Based on the results obtained and preliminary data on other common pesticides reported previously[10], it is expected that quantitative analysis using the procedure presented here can be extended to other common pesticides.

## REFERENCES

1 L. H. Keith, *Identification and Analysis of Organic Pollutants in Water*, Ann Arbor Sci. Publ., Ann Arbor, MI, 1977, p. 155.
2 Th. Noy, E. Weiss, T. Herps, H. van Cruchten and J. Rijks, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 181.
3 E. Noroozian, F. A. Maris, M. W. F. Nielsen, R. W. Frei, G. J. de Jong and U. A. Th. Brinkman, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 17.
4 A. Zlatkis, F. S. Wang and H. Shanfield, *Anal. Chem.*, 54 (1982) 2406.
5 A. Zlatkis, F. S. Wang and H. Shanfield, *Anal. Chem.*, 55 (1983) 1848.
6 N. J. Sung, S. I. Johnson, A. Zlatkis and B. S. Middleditch, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1988) 409.
7 J. P. Gibb and M. J. Barcelona, *J. Am. Water Works Assoc.*, 76 (1984) 48.

8 L. G. Bravo and L. Rejthar, *Int. J. Environ. Anal. Chem.*, 24 (1986) 305.
9 H. J. Cortes, in F. Yang (Editor), *Microbore Column Chromatography —A Unified Approach*, Marcel Dekker, New York, 1989, p. 211.
10 H. J. Cortes, C. D. Pfeiffer, G. L. Jewett and B. E. Richter, *J. Microcolumn Sep.*, 1 (1989) 28.
11 K. Grob, Jr. and B. Schilling, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 736.
12 H. J. Cortes and C. D. Pfeiffer, *Chromatogr. Forum*, 4 (1986) 29.
13 G. Takeoka and W. Jennings, *J. Chromatogr. Sci.*, 22 (1984) 177.
14 K. Grob, Jr. and B. Schilling, *J. Chromatogr.*, 39 (1987) 3.
15 S. D. Farwell, D. R. Gage and R. A. Kagel, *J. Chromatogr. Sci.*, 19 (1981) 358.

*Journal of Chromatography*, 503 (1990) 385–401
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 181

# Aluminum ion mediated stabilization of silica-based anion-exchange packings to caustic regenerants

EDWARD A. PFANNKOCH* and BARBARA S. SWITZER
*Washington Research Center, W. R. Grace & Co.-Conn., 7379 Route 32, Columbia, MD 21044 (U.S.A.)*
and
WILLIAM KOPACIEWICZ
*Amicon Division, W. R. Grace & Co.-Conn., 17 Cherry Hill Drive, Danvers, MA 01923 (U.S.A.)*
(First received September 20th, 1989; revised manuscript received November 28th, 1989)

## SUMMARY

Silica-based chromatography columns can be effectively regenerated with sodium hydroxide solutions containing millimolar levels of aluminum salts. The presence of aluminum ions in the caustic wash significantly reduced and in some cases virtually eliminated silica dissolution from silica-based anion-exchange columns. Wide-pore polymer coated anion-exchange packings for protein separations were washed in excess of 100 cycles with 0.1 *M* and 0.5 *M* sodium hydroxide containing aluminum nitrate with no detectable deterioration of the column bed or chromatographic performance.

The protection results from incorporation of aluminosilicate sites on the silica support surface beneath the polymeric coating. This aluminosilicate is insoluble in the operational pH range (5–9), therefore aluminum (as $Al_2O_3$) is undetectable at a sensitivity of 800 parts per $10^9$ in the chromatographic effluent. The protective aluminosilicate can be removed by an acid wash.

The physical/chemical changes in the column packing which result from caustic washing were studied by solid-state NMR and other physical techniques. The extent of modification, proposed protection mechanism and the influence on chromatographic performance are discussed.

## INTRODUCTION

The complexity of biological mixtures poses specific problems for the chromatographic isolation of proteins. It is not unusual for some fraction of the loaded sample mass to remain adsorbed to the column after normal operation. Depending on the sample origin, typical strongly bound materials may consist of proteins, nucleic acids, lipids, phospholipids or lipopolysaccharides. These compounds can deposit on the chromatographic surface and restrict mass transport, block adsorption sites and create non-specific binding sites.

0021-9673/90/$03.50 

In many cases column fouling can be minimized with meticulous sample preparation and/or the use of guard columns. Even with such precautions, strongly binding materials still find their way to the chromatographic column eventually leading to decreased performance and finally irreversible column deterioration. Once performance is lost, a mobile phase which both desorbs and solubilizes the deposits must be passed through the column in order for it to be recovered. Reasonably effective regenerants include solutions of high ionic strength, chaotropic agents, acids, and alcohols or other organic modifiers.

Dilute base (0.1 or 0.5 *M* sodium hydroxide) is one of the most popular cleaning agents. Its effectiveness, low cost and low toxicity are especially attractive for process chromatography columns and other equipment used in protein production. At this scale, the need to easily and effectively regenerate columns is paramount, since many of the purified proteins will be used as injectable therapeutics.

The unique properties of silica, such as high surface area, density and mechanical stability, combined with the hydrophilic polymer coating technologies now available, have led to an increased application of these packings in process operations. The use of base for regenerating silica-based chromatography columns would be preferred if silica dissolution at high pH could be minimized or eliminated.

Three general approaches have been taken to extend the useful upper pH limit of silica-based chromatographic sorbents. Polymeric organic coatings were applied to porous silicas to simultaneously confer chromatographic properties and high pH protection[1]. Silicas have been coated with various metal oxides or hydroxides to improve the pH stability of the silica support[2–4]. Silica guard columns have been used to presaturate the solvent stream with dissolved silica to suppress dissolution of the chromatographic column[5]. All of these techniques provided some enhanced pH stability above the normal upper limit of pH 8. None of these procedures, however, are effective at the pH extremes (pH 13–14) shown to be effective for cleaning anion-exchange columns.

We have shown that silica-based anion-exchange columns can be regenerated with sodium hydroxide solutions containing low levels of aluminum salts which effectively suppress silica dissolution at high pH[6]. The protection mechanism appears to result from the formation of an aluminosilicate layer on the support surface maintained by the continuous presence of aluminum ions in the sodium hydroxide solution. This aluminosilicate layer is insoluble under normal chromatographic pH conditions which therefore prevents any detectable leaching of $Al_2O_3$ during gradient operation.

## THEORETICAL

Silica can exist in several forms ranging from crystalline to amorphous, each type having a characteristic solubility in aqueous solution. Amorphous species generally have the highest solubility of all common forms of silica. Silicas used as chromatographic supports consist almost exclusively of the porous amorphous form.

Silica dissolves in aqueous solutions below pH 9 to form free monosilicic acid, $Si(OH)_4$, according to eqn. 1:

$$(SiO_2)_x + 2H_2O \rightarrow Si(OH)_4 + (SiO_2)_{x-1} \quad (1)$$

The equilibrium solubility of pure amorphous silica between pH 2 and 9 ranges between 100 and 150 ppm[7]. Above pH 10.7 monosilicic acid ionizes to form the soluble silicate[8] according to eqn. 2:

$$Si(OH)_4 + OH^- \rightleftarrows Si(OH)_5^- \qquad K_{eq} = 1.8 \cdot 10^4 \qquad (2)$$

At high pH the equilibrium strongly favors formation of the soluble silicate, reducing the concentration of monosilicic acid which leads to complete dissolution of the amorphous solid.

Because of this solubility behavior, the use of high-pH solutions for elution or cleaning of silica-based chromatography columns has been avoided. The practical upper limit of eluent pH for use on siliceous columns without significantly shortening column lifetime is approximately pH 8.5.

It is well known that low levels of certain impurities can reduce the rate of dissolution of silica as well as the solubility at equilibrium. Lewin[9] found that silica dissolution at pH 8 was retarded in the presence of salts of Al, Be, Fe, Ga, Gd, and Y. Dmitrevskii *et al.*[10] showed that the presence of $Al^{3+}$, $Ca^{2+}$ and $Mg^{2+}$ sharply reduced silica solubility in alkaline solution. Iler[2] and Lieflander and Stöber[11] have shown that when 5–50% of a silica surface is occupied by aluminosilicate the solubility falls off drastically.

This phenomenon has been put to practical use to reduce the attack of caustic wash solutions on glass. Aluminum[12], zinc[13] and beryllium[14] have been included in caustic solutions for washing soda-lime glass bottles. Of all polyvalent metals studied, aluminum appears to have the most dramatic impact for suppressing silica solubility.

The nature of "aluminate" solutions has been studied by Raman spectroscopy, $^{27}Al$ NMR spectroscopy and ion-exchange chromatography. From pH 8 to 12 the principal aluminum species appears to be a polymer with octahedral Al and OH bridges[15]. Above pH 13 the tetrahedral $Al(OH)_4^-$ predominates and is formed from aluminum salts including the nitrate, sulfate, chloride and acetate according to eqn. 3:

$$Al^{3+} + 4OH^- \rightleftarrows Al(OH)_4^- \qquad (3)$$

The aluminate ion, $Al(OH)_4^-$ is geometrically similar to the tetrahedrally coordinated silicon in a silica surface. It is postulated that this facilitates insertion or exchange into the $SiO_2$ surface creating an aluminosilicate site having a fixed negative charge[2]. Iler has proposed that these fixed anionic sites repel the approach of hydroxyl ions in solution, thus reducing the rate of dissolution of silica.

The equilibrium solubility of silica in the presence of aluminate ion is also substantially reduced. This is found to be the case even when the silica surface contains as little as 5% aluminosilicate[2]. The aluminosilicate itself is known to be quite insoluble; however the mechanism by which such low level incorporation can suppress silica solubility is not well understood.

## EXPERIMENTAL

### *Materials*

PAE-1000 packing and uncoated 1000-Å pore diameter silica (both 10 μm) were from Amicon (Danvers, MA, U.S.A.). Bovine serum albumin (BSA, fraction V powder) and ovalbumin (OVA, grade V) were from Sigma (St. Louis, MO, U.S.A.). Tris(hydroxymethyl)amino methane, sodium hydroxide, sodium chloride, and aluminum nitrate [$Al(NO_3)_3 \cdot 9H_2O$] were from J. T. Baker (Phillipsburg, NJ, U.S.A.) and were of the highest grade available. HPLC-grade deionized water was produced by using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

### *Apparatus*

*Dynamic solubility test.* Two systems were operated in parallel, each consisting of an Altex 110B pump (Beckman, Palo Alto, CA, U.S.A.) and an LKB 2112 Redirac fraction collector (Pharmacia, Piscataway, NJ, U.S.A.).

*Column longevity model system —cycle test.* Two chromatographic systems were configured to carry out automated model studies of column longevity. System 1 was controlled by a Waters Model 840 data station (DEC Pro 350 based) and consisted of two M510 pumps, a refrigerated WISP 712 autosampler and a Model 490 variable-wavelength programmable UV detector operating at 280 nm. The chromatographic gradient was monitored by using an Anspec Model AN-400 conductivity detector (Anspec, Ann Arbor, MI, U.S.A.) in-line after the UV detector. An Autochrome Model 101 3-solvent select valve (Autochrome, Milford, MA, U.S.A.) was installed on the inlet line of the "B pump" to allow computer selection of strong buffer, caustic or deionized water.

System 2 consisted of a Waters 720 system controller, two Waters M6000A pumps, a refrigerated WISP 712 autosampler and a Kratos Spectroflow 757 variable-wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) operating at 280 nm. An Autochrom 3-solvent select valve was installed on the inlet line of the "B pump" as in system one. The chromatographic gradient was monitored by using an in-line Anspec AN-400 conductivity detector after the UV detector, and both detector outputs were monitored simultaneously by using a Waters data module.

*BSA frontal uptake.* All BSA frontal uptake experiments to determine column loading capacity for proteins were carried out on a Waters liquid chromatograph consisting of a Model 720 system controller, two M6000A pumps, a Kratos Spectroflow 757 variable-wavelength UV detector operating at 288 nm and a Waters data module. The protein solution (BSA, 2.5 mg/ml) was loaded onto the column through an Altex 110B pump plumbed into the system via a Valco 6-port sample valve (Valco, Houston, TX, U.S.A.).

### *Methods*

*Column packing.* Stainless-steel columns (25 × 0.41 cm I.D.) were packed by using a Haskel pump and an upward stirred slurry technique. Packing material was slurried in 30 ml of 2-propanol and packed at 5000 p.s.i. with methanol as the packing solvent. Columns were flushed with deionized water before use.

*Dynamic solubility test.* Stainless-steel columns (25 × 0.41 cm I.D.) packed with

PAE-1000 sorbents were flushed at 1 ml/min with 15 ml of 0.5 *M* sodium hydroxide and kept at room temperature for 4 h to precondition the column. Caustic solutions containing various levels of aluminum nitrate (such that the final sodium hydroxide concentration was either 0.1 or 0.5 *M*) were then pumped through the column at 1 ml/min collecting fractions of 5.5 ml. Fractions 2–8 were diluted 1:1 with deionized water and analyzed for silica by atomic absorption spectroscopy. It was empirically determined that the silica level seen in fractions 6–8 represents a plateau which predicts with reasonable accuracy the silica level obtained while washing with caustic during an automated 7-day column longevity study.

*Column longevity model system —cycle test.* The design of the automated cycle test models extended column lifetime under rigorous use conditions. The key elements of the test include (1) a chromatographic separation of BSA and OVA on a 25 × 0.41 cm I.D. column, (2) a caustic wash of specified duration, and (3) readjustment of column pH in preparation for the next chromatographic cycle. The actual hardware connections and software details are dependent upon the high-performance liquid chromatography (HPLC) system used, therefore only a general description of the test system is given.

The system was initially set up with 0.01 *M* Tris–HCl of pH 8 in pump A. The three solvent lines for pump B contained (A) 0.1 *M* Tris–HCl + 0.5 *M* sodium chloride, pH 8, (B) caustic (as specified), and (C) deionized water. A sample mixture containing 6.2 mg/ml BSA and 4.9 mg/ml OVA was maintained at 4°C in the autosampler.

The system was programmed to carry out a 20-min linear gradient from 100% A to 100% B, followed by a 5-min hold at 100% B. At 25 min, a signal from the data system advanced the solvent select valve, starting the caustic wash. The duration of the caustic wash depended on the caustic concentration: 4 column volumes of 0.5 *M* caustic or 10 column volumes of 0.1 *M* caustic. At the end of the caustic wash the solvent select valve was again advanced, and 2 ml of deionized water was pumped through the column. The solvent select valve was again advanced equilibrating the column for 10 min with buffer B, after which the chromatographic system was equilibrated for 15 min with buffer A in preparation for the next chromatographic run. A schematic of the cycle test is shown in Fig. 1.

Injections (50 μl) of the BSA–OVA mixture were repeated over the course of the test totalling 100 cycles. Resolution ($R_s$) of the BSA–OVA mixture was calculated according to eqn. 4 and plotted *vs.* cycle number.

$$R_s = \frac{2(t_2 - t_1)}{\Delta t_1 + \Delta t_2} \tag{4}$$

In this equation $t_1$ and $t_2$ are the retention times of OVA and BSA respectively, and $\Delta t_1$ and $\Delta t_2$ are their peak widths at the baseline. Early column failure was indicated by a loss of resolution and a catastrophic column bed collapse.

*BSA frontal uptake measurement.* Stainless-steel columns (25 × 0.41 cm I.D.) containing PAE-1000 packing were washed at 1 ml/min with 20 ml of 0.01 *M* Tris–HCl + 1 *M* sodium chloride, pH 8, then equilibrated in 0.01 *M* Tris–HCl, pH 8. By rotating the Valco 6-port valve, a BSA solution (2.5 mg/ml in 0.01 *M* Tris–HCl, pH 8) was pumped onto the column at 1 ml/min through an Altex 110B pump. The column

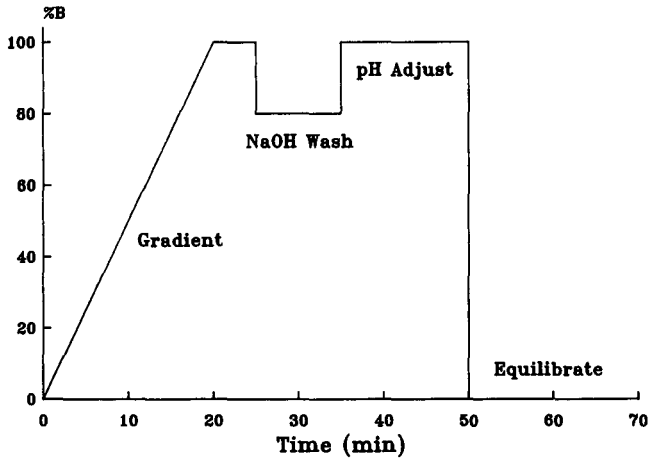



Fig. 1. Schematic representation of the segments comprising one cycle of the automated column longevity system. One longevity test consisted of 100 cycles carried out continuously for 7 days.

effluent was monitored at 288 nm to assure that the absorbance of the BSA solution remained on scale at a detector setting of 1.0 a.u.f.s. Column saturation was judged when the detector output was 20% of the full-scale BSA absorbance. Volumetric and mass loading capacities were determined from the calculated mass of BSA bound and the column volume (3.3 ml) or mass of packing material (1.3 g packing/column).

## RESULTS AND DISCUSSION

### *Aluminum-ion mediated suppression of silica solubility*

To accurately assess the effect of increasing aluminum ion concentration on silica solubility in a chromatographic column, a non-equilibrium test system was employed. Shaker-flask type equilibrium solubility tests for silica in the presence of aluminum ion predict erroneously low silica solubility levels compared to silica levels actually observed in a pumped system.

Aluminum nitrate-containing sodium hydroxide wash solutions were prepared to give the final $Al^{3+}$ and $OH^-$ concentrations indicated in Table I allowing for the consumption of $OH^-$ by $Al^{3+}$ as shown in eqn. 3. If this allowance is not made, the final solution pH can fall below 13 and precipitation of $Al_2O_3$ can occur.

Control columns washed with sodium hydroxide solutions containing no aluminum salts exhibited high concentrations of $SiO_2$ in the column effluent. Columns washed with caustic solutions containing high aluminum nitrate levels exhibited significantly lower $SiO_2$ dissolution than controls. Since our purpose was to determine the lowest practical aluminum concentration needed to effectively protect the silica for a minimum of 100 wash cycles, the range of 0.005–0.01 *M* $Al^{3+}$ was determined to be optimal for these adsorbents. In addition to aluminum nitrate, we have studied other aluminum salts including the chloride, acetate and sulfate and found no difference in their ability to suppress silica solubility[6]. Gallium chloride also suppressed silica solubility, although less effectively (data not shown). An alumina-containing pre-column was also used effectively as an aluminum ion source to suppress silica

TABLE I

DYNAMIC SOLUBILITY TEST RESULTS —PAE 1000

Data were collected as described in Experimental. Silica values represent the average of two separate determinations. Increasing aluminum nitrate concentration reduces the dissolved silica levels observed in the column effluent.

| *[$Al^{3+}$] (mol/l)* | *0.1 M NaOH + 1.0 M NaCl, ppm $SiO_2$* | *0.5 M NaOH, ppm $SiO_2$* |
|---|---|---|
| 0 | 250 | 480 |
| 0.002 | 110 | 320 |
| 0.005 | 52 | 130 |
| 0.01 | 43 | 120 |
| 0.02 | 33 | 80 |
| 0.04 | 33 | 80 |
| 0.08 | 19 | 51 |

dissolution. No attempt was made to optimize precolumn or alumina particle dimensions (data not shown).

To maintain efficient suppression of silica solubility at high pH the continuous presence of aluminum ions in the caustic solution is required. A column was flushed with 1 l of caustic containing aluminum nitrate until silica dissolution was significantly reduced (Fig. 2A). The wash solution was then changed to 0.1 *M* sodium hydroxide. Removal of the aluminum salt from the caustic wash resulted in a rapid stripping of adsorbed aluminum from the silica surface and a concomittant increase in $SiO_2$ levels (Fig. 2B).

*Column longevity studies*

Column longevity was assessed as a function of repeated caustic wash cycles. For this purpose, an automated test system which would model column conditions during preparative chromatography was designed. Column performance was judged during each chromatographic cycle by monitoring the separation of a protein test mixture containing BSA and OVA. Fractions (15 ml) were collected during the chromatographic gradient and caustic wash portions of the cycle for determination of $SiO_2$ and $Al_2O_3$ levels.

In this test system, each chromatographic separation was followed by a caustic wash cycle. In practice, column washing may be done much less frequently depending on the sample composition, the tendency toward column fouling, and the stringency of the validation procedures. Two different wash protocols using 0.1 and 0.5 *M* sodium hydroxide were chosen to best represent the range of conditions typically used to clean preparative chromatography columns.

*0.1 M sodium hydroxide wash*

As a control, PAE-1000 columns were washed with 10 column volumes of a solution containing 0.1 *M* sodium hydroxide + 1.0 *M* sodium chloride. The silica level during the caustic wash increased to approximately 400 ppm before bed collapse between cycle 30 and 40 (Fig. 3A). This represents dissolution of approximately 30% of the silica support during the wash cycles. Total wash volume before bed collapse was

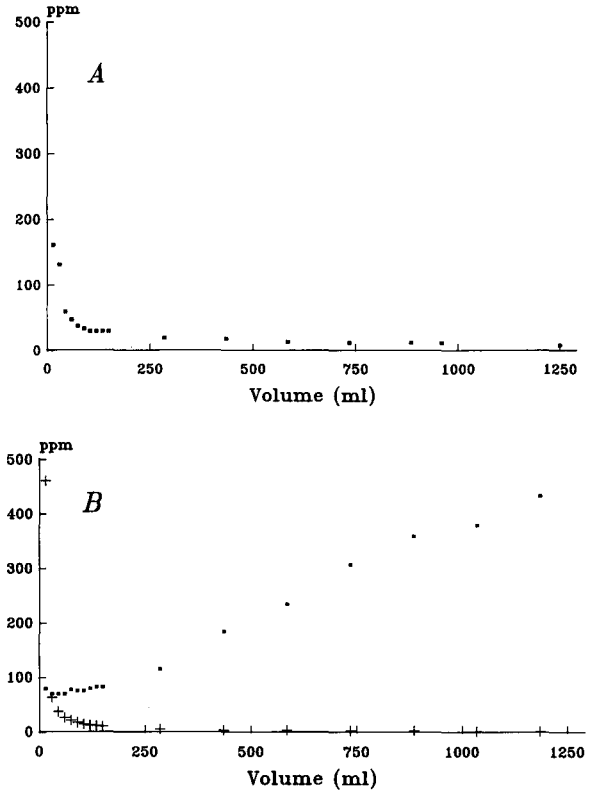



Fig. 2. (A) Dynamic solubility test results for PAE-1000 column (250 × 4.1 mm I.D.) washed at 1 ml/min with 0.18 *M* sodium hydroxide + 0.02 *M* aluminum nitrate. For details of test conditions, see Experimental. Silica solubility (in ppm) decreased sharply as the protective aluminosilicate layer formed. (B) Dynamic solubility test results obtained when aluminum-containing sodium hydroxide wash solution was replaced by 0.1 *M* sodium hydroxide. Accumulated aluminosilicate was rapidly lost and silica solubility increased steadily to levels comparable to untreated silica. ■ = $SiO_2$; + = $Al_2O_3$.

approximately 1 l. Silica levels during the chromatographic gradient were approximately 12 ppm. A large void (*ca.* 10 cm) was formed at the head of the 25-cm column after bed collapse. It is noteworthy that column performance as measured by BSA–OVA resolution remained reasonably constant until shortly before bed collapse (Fig. 3B).

Silica levels during the caustic wash were reduced to approximately 25 ppm throughout the entire 100-cycle test for PAE-1000 columns washed with caustic containing optimized aluminum nitrate levels (Fig. 4A). Furthermore, silica levels during the chromatographic gradient were reduced to approximately 2 ppm and aluminum (as $Al_2O_3$) was undetectable in the gradient fractions at 800 ppb[a] sensitivity. Column performance as measured by resolution of the BSA–OVA mix was unchanged through 100 cycles (Fig. 4B). No evidence for void formation at the inlet was observed in either column.

[a] Throughout this article, the American billion ($10^9$) is meant.

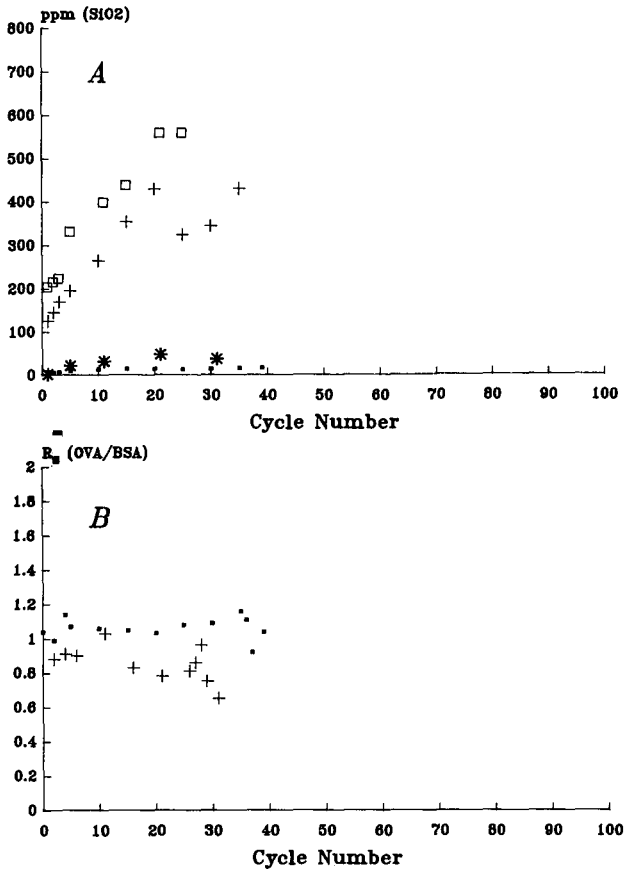



Fig. 3. Cycle test results for control PAE-1000 columns (250 × 4.1 mm I.D.) washed with 10 column volumes of 0.1 *M* sodium hydroxide + 1.0 *M* sodium chloride after each chromatographic run. Different symbols indicate results of duplicate experiments. For details of cycle test conditions, see Experimental. (A) Silica level during caustic wash (□, +) and chromatographic gradient (■, ∗). (B) Resolution ($R_s$) of BSA–OVA mixture for duplicate experiments (■, +).

Cycle testing of PAE-1000 under these optimized conditions was repeated for extended periods until eventual column failure (data not shown). Chromatographic performance was maintained unchanged through 300 cycles. Gradual deterioration in performance, probably due to channeling in the column bed, was seen from that point until complete bed collapse occurred at cycle 425. Put in perspective, this represents washing a 25 × 0.41 cm I.D. analytical column with approximately 14 l of 0.1 *M* sodium hydroxide. At the end of the test, approximately 40% of the silica support had been dissolved.

*0.5 M sodium hydroxide wash*

PAE-1000 columns were washed with 4 column volumes of 0.5 *M* sodium hydroxide or 0.5 *M* sodium hydroxide containing aluminum nitrate (Figs. 5 and 6). Despite the higher pH wash, control columns exposed to a 4-column-volume wash

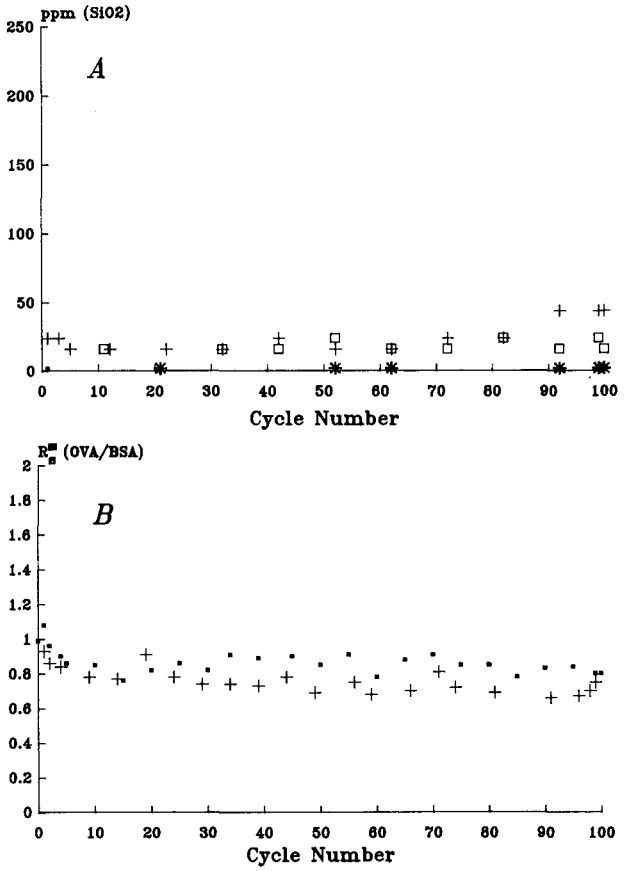



Fig. 4. Cycle test results for duplicate PAE-1000 columns (250 × 4.1 mm I.D.) washed with 10 column volumes of 0.12 *M* sodium hydroxide + 0.005 *M* aluminum nitrate + 1.0 *M* sodium chloride. (A) Silica level during caustic wash (□, +) and chromatographic gradient (■, ∗). (B) Resolution ($R_s$) of BSA–OVA mixture for duplicate experiments (■, +).

survived over 50 cycles as compared to 36 cycles for controls washed with 10 column volumes of 0.1 *M* sodium hydroxide + 1.0 *M* sodium chloride (Fig. 5A). This again represents dissolution of approximately 30% of the silica support. Column performance was maintained until shortly before bed collapse (Fig 5B). The presence of aluminum nitrate significantly reduced silica levels during both the caustic wash and chromatographic gradient (Fig. 6A). Column performance remained unchanged throughout the 100-cycle test (Fig. 6B), and there was no evidence for void formation at the column inlet. Chromatographic peak shape was maintained throughout the cycle test (Fig. 7).

Extended cycle testing of PAE-1000 washed with 0.5 *M* sodium hydroxide containing aluminum nitrate was repeated until eventual column failure (data not shown). Chromatographic performance remained unchanged through over 200 wash cycles. Noticeable column deterioration then progressed until complete bed collapse occurred at cycle 275. Based on dissolved silica in the wash solutions, approximately 40% of the silica support had been dissolved before bed collapse.

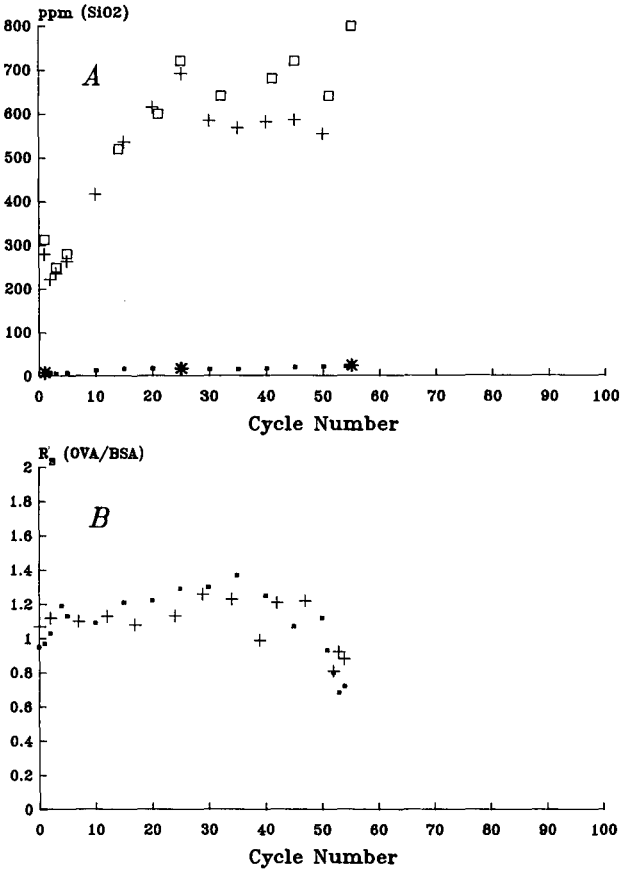



Fig. 5. Cycle test results for duplicate control PAE-1000 columns (250 × 4.1 mm I.D.) washed with 4 column volumes of 0.5 *M* sodium hydroxide after each chromatographic run. (A) Silica level during caustic wash (□, +) and chromatographic gradient (■, ∗). (B) Resolution ($R_s$) of BSA–OVA mixture for duplicate experiments (■, +).

For comparison, cycle testing was carried out by using less than optimal wash conditions, *i.e.* 0.002 *M* aluminum nitrate in 0.5 *M* sodium hydroxide. The column showed no evidence of deterioration during the 100-cycle test. The silica solubility data in Table I suggests that an extended test would show a reduced total lifetime compared with the above test using optimal aluminum nitrate levels.

The fact that aluminate ion exists in solution above pH 13 as the tetrahedral $Al(OH)_4^-$ is crucial to the use of aluminum salts as additives in caustic wash solutions for silica-based anion-exchange packings. Below pH 13, polymeric alumina predominates and can precipitate, rapidly poisoning the anion-exchange column (data not shown). Above pH 13 the $Al(OH)_4^-$ monomer predominates and has no deleterious effect on either the adsorbed, cross-linked organic coating or the chromatographic performance. The $Al(OH)_4^-$ apparently can diffuse through the organic coating and exchange into the underlying silica surface to form a protective aluminosilicate layer. This significantly reduces silica dissolution, allowing repeated cleaning with caustic containing aluminum salts with no significant deterioration in column performance.

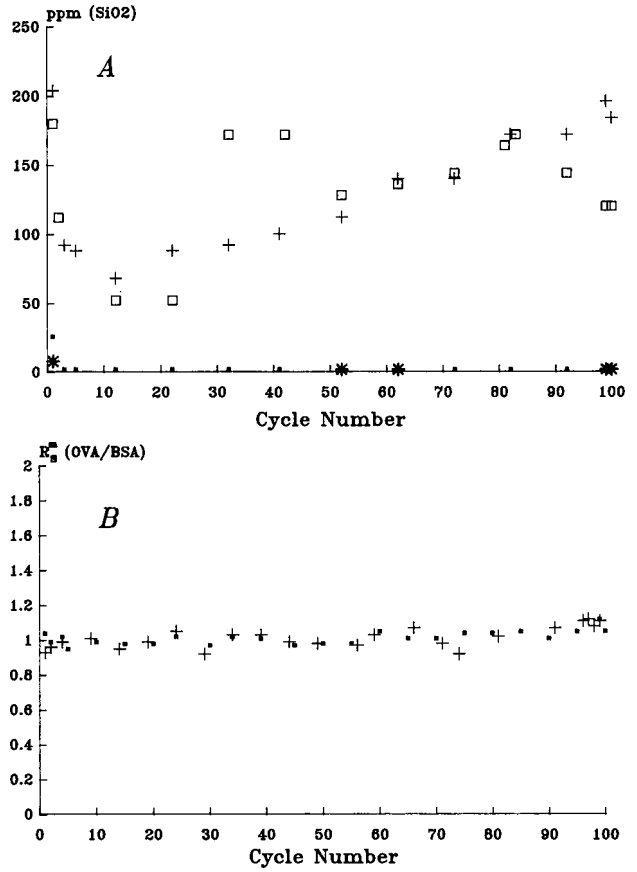



Fig. 6. Cycle test results for duplicate PAE-1000 columns (250 × 4.1 mm I.D.) washed with 4 column volumes of 0.52 *M* sodium hydroxide + 0.005 *M* aluminum nitrate. (A) Silica level during caustic wash (□, +) and chromatographic gradient (■, ∗). (B) Resolution ($R_s$) of BSA–OVA mixture for duplicate experiments (■, +).

*Characterization of the aluminosilicate layer*

To investigate the physical/chemical changes occurring on the silica support after exposure to caustic solutions containing aluminum nitrate, sorbent was recovered from the column and analysed by magic angle spinning solid state $^{27}Al$ NMR. The presence of Al–O–Si bonds from an aluminosilicate was confirmed, therefore the aluminum appears to be chemically incorporated into the silica surface as opposed to being simply chemisorbed upon the surface[16].

Studies were carried out to determine the extent of $Al_2O_3$ incorporation into the support during the course of the cycle test. For this purpose, 25 × 0.41 cm I.D. columns of PAE-1000 were washed at 1 ml/min with volumes of 0.12 *M* sodium hydroxide + 1 *M* sodium chloride + 0.005 *M* aluminum nitrate corresponding to 1, 5, 10 or 100 wash cycles. After an exhaustive water wash to remove unbound aluminum, the silica was recovered from the column, dried and weighed. Weight percent aluminum was determined by atomic absorption spectroscopy after digestion with hydrofluoric acid–perchloric acid.

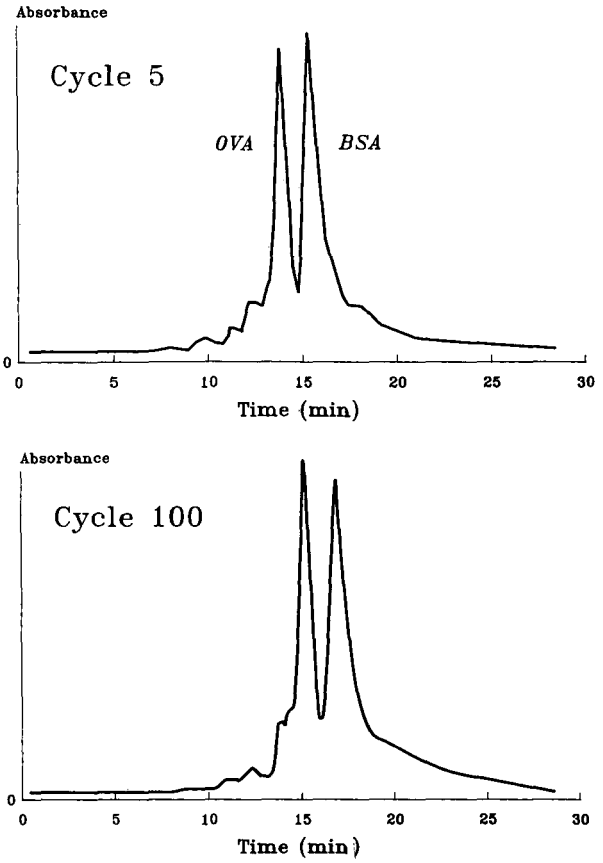



Fig. 7. Representative chromatograms from the beginning and end of a cycle test with aluminum nitrate-containing caustic. Column, 25 × 0.41 cm I.D. PAE-1000; wash, 4 column volumes of 0.52 *M* sodium hydroxide + 0.005 *M* aluminum nitrate; gradient, as described in Experimental section.

TABLE II

BSA FRONTAL LOADING CAPACITY ON PAE-1000 COLUMNS

Data were collected as described in Experimental. Column loading capacity values were the result of at least two determinations. No significant change in loading capacity was seen for columns exposed to 100 wash cycles relative to controls.

| *Treatment* | *BSA loading capacity* | | |
|---|---|---|---|
| | *mg/column* | *mg/ml* | *mg/g packing* |
| None | 145 | 44 | 112 |
| 110 cycles 0.12 *M* NaOH + 0.005 *M* $Al(NO_3)_3$ + 1 *M* NaCl | 155 | 47 | 120 |
| 100 cycles 0.52 *M* NaOH + 0.005 *M* $Al(NO_3)_3$ | 139 | 42 | 106 |

The protective aluminosilicate layer was found to form rapidly with greater than 50% deposited in the first cycle, and 67% adsorbed by cycle 10. The final level incorporated (0.33%, w/w) represents 1.6 aluminum atoms/nm$^2$ which corresponds to 20% of a monolayer assuming 8 metal atom sites per nm$^2$ (ref. 2). This number agrees well (perhaps coincidentally) with the maximum aluminum incorporation seen by Iler[2] for colloidal silica at pH 8.

Iler[2] suggested that the rate of silica dissolution was suppressed in the presence of aluminum ions by the formation of negatively charged aluminosilicate sites which repel the approach of hydroxyl ions. Adsorbed aluminum also reduced the equilibrium solubility of the silica despite formation of less than a complete monolayer. One might speculate that through the dynamic equilibrium process, the presence of aluminosilicate sites creates a short range order in the silica surface, reducing the equilibrium solubility in high-pH solutions. We found no evidence for significant crystallinity detectable by X-ray diffraction techniques in these and related materials[17].

Several acids were evaluated as stripping agents for those cases in which trace aluminum buildup on-column is to be avoided. Both 0.1 *M* hydrochloric acid and 0.1 *M* orthophosphoric acid were quite effective at removing bound alumina from columns exposed to 100 caustic wash cycles. Phosphoric acid, being less corrosive to stainless steel systems was the method of choice. A 30-ml wash of 0.1 *M* orthophosphoric acid removed approximately 85% of the accumulated aluminum from a 25 × 0.46 cm I.D. column. For all practical purposes, however, since the aluminum leaching level during the chromatographic gradient is less than 800 ppb, acid stripping should not be required after caustic washing.

*BSA loading capacity studies*

Although extensive cycle testing with aluminum-containing caustic showed no evidence for performance deterioration of PAE-1000 with regard to the separation of BSA and OVA, this did not necessarily demonstrate bonded phase integrity. Gradient elution protein chromatography is known to be sensitive to column bed integrity and channeling but relatively insensitive to column length[18] and hence the mass of functional sorbent. It may therefore be possible to strip a substantial fraction of the bonded phase from the column without significantly affecting analytical scale column performance.

BSA frontal loading capacity studies were carried out on PAE-1000 columns before and after exposure to 100 caustic wash cycles to demonstrate conclusively that washing with caustic containing aluminum nitrate did not promote bonded phase loss. BSA frontal loading capacity was determined before and after cycle testing as described in materials and methods. BSA does not bind to uncoated silica sorbents under the specified anion-exchange chromatography conditions. A loss of the coating would be detected by a reduction in BSA loading capacity. Column loading capacity remained virtually unchanged after exposure to 100 caustic cycles (Table II), therefore no significant bonded phase loss occurred.

PAE-1000 packings were prepared by adsorption and subsequent cross-linking of cationic polymers[1]. This chemistry produces a continuous pellicular coating which is quite resistant to deterioration at high pH. Silica-based anion-exchange packings for protein separations can also be prepared by covalent attachment of organosilanes[19] or preformed cationic polymers[20,21]. Caustic attack rapidly strips covalent bonded

phases (data not shown), whereas slow dissolution of the silica from beneath an adsorbed, cross-linked coating can proceed up until bed collapse with virtually no effect on the chromatographic performance.

*Physical changes due to caustic washing*

Cycle test results showed no detectable deterioration in chromatographic performance after 100 washes with aluminum-containing caustic. However, silica levels during the caustic wash indicated that some minor erosion of the inorganic support did occur. Long-term cycle tests also showed that columns eventually did collapse due to removal of approximately 40% of the silica support. Pore volume, pore diameter and surface area measurements were made on sorbents recovered after cycle testing to determine whether physical changes in the support could be detected after 100 wash cycles.

Washing with caustic (either 0.1 *M* or 0.5 *M* sodium hydroxide) caused rapid bed collapse in control experiments due to removal of silica which in turn weakened the bed structure (Table III). A 45–60% increase in pore volume and up to 2-fold increase in surface area was observed relative to untreated packing material. An overall decrease in average pore diameter was also seen which is due to the creation of a larger population of small pores, rather than loss of large pores.

TABLE III

PHYSICAL CHANGES IN PAE-1000 AFTER CAUSTIC WASHING

Packing materials were recovered from the columns, washed to neutrality by repeated suspension and settling in deionized water, then dried under vacuum. The organic coating was pyrolized in air by a stepwise process to prevent particle fracture. Surface area and pore diameter were then determined by standard nitrogen BET and mercury porosimetry techniques.

| *Treatment* | *Pore volume* ($cm^3/g$) | *Nominal pore* | |
|---|---|---|---|
| | | *diameter* (Å) | *surface area* ($m^2/g$) |
| None (control) | 0.86 | 590 | 47 |
| 31 cycles<br>0.1 *M* NaOH + 1 *M* NaCl (collapsed) | 1.25 | 520 | 73 |
| 100 cycles<br>0.12 *M* NaOH + 1 *M* NaCl + 0.005 *M* $Al(NO_3)_3$ | 0.86 | 590 | 47 |
| 425 cycles<br>0.12 *M* NaOH + 1 *M* NaCl + 0.005 *M* $Al(NO_3)_3$ (collapsed) | 1.22 | 520 | 64 |
| 54 cycles<br>0.5 *M* NaOH (collapsed) | 1.36 | 470 | 95 |
| 100 cycles<br>0.52 *M* NaOH + 0.005 *M* $Al(NO_3)_3$ | 1.18 | 560 | 57 |

Washing with aluminum nitrate-containing 0.1 *M* sodium hydroxide for 100 cycles resulted in virtually no change in the physical characteristics of the support. This would suggest very effective suppression of silica dissolution under the chosen wash conditions, which was confirmed by the 425-cycle lifetime seen in extended tests. Sorbent recovered after bed collapse following 425 wash cycles showed pore volume, surface area and pore diameter changes very similar to those for control columns washed with caustic alone until bed collapse.

Washing with aluminum nitrate-containing 0.5 *M* sodium hydroxide for 100 cycles caused alterations in pore volume, surface area and pore diameter intermediate between those seen for untreated and collapsed sorbents. This is reasonable based upon the dissolved silica level predicted by dynamic solubility tests and seen during caustic washing in cycle tests, as well as the lower lifetime (225 cycles) relative to 0.1 *M* sodium hydroxide washing. If additional stability is desired using 0.5 *M* sodium hydroxide wash solutions, the aluminum nitrate concentration can be increased based upon the silica solubility data shown in Table I.

## CONCLUSIONS

The addition of millimolar amounts of aluminum salts to sodium hydroxide wash solutions above pH 13 significantly suppressed silica dissolution from silica-based anion-exchange packings. A constant source of aluminum must be present during the high pH wash to provide continuous protection of the silica support. Chromatographic performance, the major determinant of column lifetime, is virtually unaffected by slow dissolution of the silica support beneath the polymeric anion-exchange coating until shortly before column bed collapse.

BSA loading capacity analysis before and after cycle testing showed that the adsorbed polymeric anion-exchange coating used to prepare PAE-1000 packings was stabile through at least 100 aluminum nitrate-containing caustic wash cycles. Column performance as determined by the chromatographic resolution of BSA and OVA remained unchanged for 200–300 caustic wash cycles depending upon the sodium hydroxide concentration. This represents up to a 10-fold improved column lifetime relative to control columns washed with aluminum-free sodium hydroxide. Covalent chromatographic coatings showed significant deterioration when washed with caustic solutions containing aluminum nitrate, most likely due to the cleavage of the organosilane linkage.

Solid-state $^{27}Al$ NMR identified the presence of an aluminosilicate layer on the silica support after exposure to caustic solutions containing aluminum nitrate. The amount of alumina accumulated after 100 wash cycles represented less than a monolayer, yet provided sigificant suppression of silica solubility. Adsorbed aluminum did not appear to leach from the column during gradient elution at the 800 ppb detection level. The trace level of insoluble aluminosilicate on the chromatographic support can be significantly reduced by a brief wash with 0.1 *M* phosphoric acid.

The unique properties of silica-based anion-exchange sorbents can be used to their full advantage in process chromatographic applications if aluminum-containing caustic washes are employed. Processes will benefit from improved throughput and resolution as well as the ability to regenerate the column with dilute caustic solutions.

This technique may have additional applications. High-pH analytical separations may be possible by including low levels of aluminum salts in the chromatographic eluent. This is of particular interest for reversed-phase separations of basic compounds.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. M. T. Nemeth, J. P. Slanga, A. L. Roe, N. P. Luthra, M. P. Shatlock, S. Fulton and R. Kumar for their helpful discussions and technical contributions throughout this project. The authors also wish to thank Mr. L. McLaughlin for his dedicated and valuable technical assistance during the early stages of this work, and Ms. J. Whitecavage and Mr. M. Juskelis for their excellent technical support in all elemental and physical analyses.

REFERENCES

1 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
2 R. K. Iler, *J. Colloid Interface Sci.*, 43 (1973) 399.
3 R. W. Stout, *U.S. Pat.*, 4 600 646 (1986).
4 A. Barkatt and P. B. Macedo, *U.S. Pat.*, 4 648 975 (1987).
5 J. G. Atwood, G. J. Schmidt and W. Slavin, *J. Chromatogr.*, 171 (1979) 109.
6 E. A. Pfannkoch, *U.S. Pat.*, 4 808 233 (1989).
7 R. K. Iler, *The Chemistry of Silica*, Wiley, New York, 1979, p. 42.
8 R. K. Iler, *The Chemistry of Silica*, Wiley, New York, 1979, p. 47.
9 J. C. Lewin, *Geochim. Cosmochim. Acta*, 21 (1961) 182.
10 G. E. Dmitrevskii, L. G. Martynova, E. I. Antoshenko and Z. T. Lukomskaya, *Zh. Prikl. Khim. Leningrad*, 44 (1971) 2381.
11 M. Lieflander and W. Stöber, *Z. Naturforsch. B*, 15 (1960) 411.
12 W. C. Cooper, *U.S. Pat.*, 2 241 984 (1941).
13 W. F. Wegst, L. R. Bacon and T. H. Vaughn, *U.S. Pat.*, 2 447 297 (1948).
14 W. F. Wegst, L. R. Bacon and T. H. Vaughn, *U.S. Pat.*, 2 419 805 (1947).
15 F. A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry*, Wiley, New York, 1972, p. 267.
16 M. P. Shatlock, personal communication.
17 A. L. Roe, personal communication.
18 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 109 (1980) 345.
19 Y. A. Eltekov, A. V. Kiselev, T. D. Khokhlova and Y. S. Nikitin, *Chromatographia*, 6 (1973) 187.
20 S. Gupta, E. Pfannkoch and F. E. Regnier, *Anal. Biochem.*, 128 (1983) 196.
21 H. E. Ramsden, *U.S. Pat.*, 4 540 486 (1985).

*Journal of Chromatography*, 503 (1990) 403–409
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 216

# Product purity as a function of elution order[a]

JOHN A. PERRY* and JOHN D. RATEIKE
*Regis Chemical Company, 8210 Austin Avenue, Morton Grove, IL 60053 (U.S.A.)*
(First received May 10th, 1989; revised manuscript received November 29th, 1989)

SUMMARY

Elution order preference in preparative liquid chromatography can be examined if the elution order can be isolated as a variable, as it can with Pirkle-concept chiral stationary phases. With these phases, the elution order can be exactly reversed by replacing a column that contains a given chiral stationary phase by its enantiomeric twin.

This reversal was exhaustively replicated in a set of preparative liquid chromatographic separations (shown herein) of a chiral alcohol.

These data suggested to us that in preparative liquid chromatographic purification of a given major enantiomer, the trace enantiomer should be eluted first.

## INTRODUCTION

Should a component to be purified by preparative liquid chromatography be eluted before or after a contaminating trace?

Definitive experimentation on elution order choice[1] can be achieved only by those chiral methods that are based on Pirkle-concept chiral stationary phases (CSPs)[2–7]. In these, the elution order of two enantiomers can be exactly reversed: a first column that contains a given CSP is substituted for a second column, identical to the first, except that the CSP of the second column is the enantiomer of the CSP in the first.

With good success in each of two reported works, the trace component was eluted before the major[8,9]. In the first, an enantiomer was brought to 99.9967% enantiomeric purity[8]; in the second, the methodology was desribed for measuring that enantiomeric purity to that precision, with only conventional equipment[9].

In this paper, we describe the experimentation that underlay chiral purification and analysis, and present chromatographic evidence that caused us to elect trace-first elution as the elution order of choice.

[a] Presented at the *6th International Symposium on Preparative Chromatography, Washington, DC, May 8–10, 1989*. The majority of the papers presented at this symposium have been published in *J. Chromatogr.*, Vol. 484 (1989)

0021-9673/90/$03.50 

## EXPERIMENTAL

### *Materials*

For this study, trifluoro-1-(9-anthryl)-ethanol was used. The starting material (the racemate and its ketone precursor) had been kindly supplied by Dr. W. Pirkle of the University of Illinois. HPLC-grade solvents were used throughout.

### *Columns, equipment*

The analytical and preparative columns were laboratory-packed and are commercially available from Regis. The columns and the equipment used to pack and test them have been described[8,9].

### *Procedures*

The mobile phase used for both analytical and preparative chromatograms was hexane–isopropanol (90:10, v/v). The concentration of racemate charged to the preparative columns was 5 mg/ml of mobile phase; the concentrations in fractions were as collected.

## RESULTS AND DISCUSSION

Our experiments, strictly exploratory, were undertaken in 1984 partly to demonstrate the utility of being able to treat and control elution order as an independent variable, partly to determine the answers to questions about how best to conduct preparative liquid chromatography, and partly to determine whether a contaminating trace (here, the minor enantiomer) should be eluted before or after the main component (here, the major enantiomer). In this paper, we consider only the last aspect.

The alcohol concentrations injected could have been at least 10 times greater. However, they were deliberately restricted enough to allow the initial trace peak to be at least partially resolved from the major. We have not conducted experiments similar to these at concentrations near saturation, wherein the preparative peak would be one undifferentiated mass, and thus our conclusions here do not necessarily apply to such a case.

The chromatograms shown in Figs. 1 and 2 were not informative in choosing a preferred elution order. However, those in Figs. 3 and 4 were more helpful; in these, the first-eluted trace can be seen concentrated in the earlier fractions. Fig. 3 was particularly instructive.

In Fig. 3, the sequence of trace-first analytical chromatograms shows the trace highly concentrated in fraction 4 which, as shown in the corresponding preparative chromatogram, represented nearly the whole of the partly resolved initial enantiomeric peak. Fractions 3 and 5 contained most of the rest of the trace. The presence of the trace in fraction 6, as the major peak was entered, was barely detectable, and the trace was not detected in any later fractions.

On the other hand, in Fig. 3 the sequence of trace-last analytical chromatograms shows not only no purification whatsoever of the major component but also an unexpected and puzzling oscillation of trace concentrations throughout the major peak. (We comment further on this oscillation in the next paragraph.) That the preferred elution order is or should be trace-last[1], conflicts with these data.

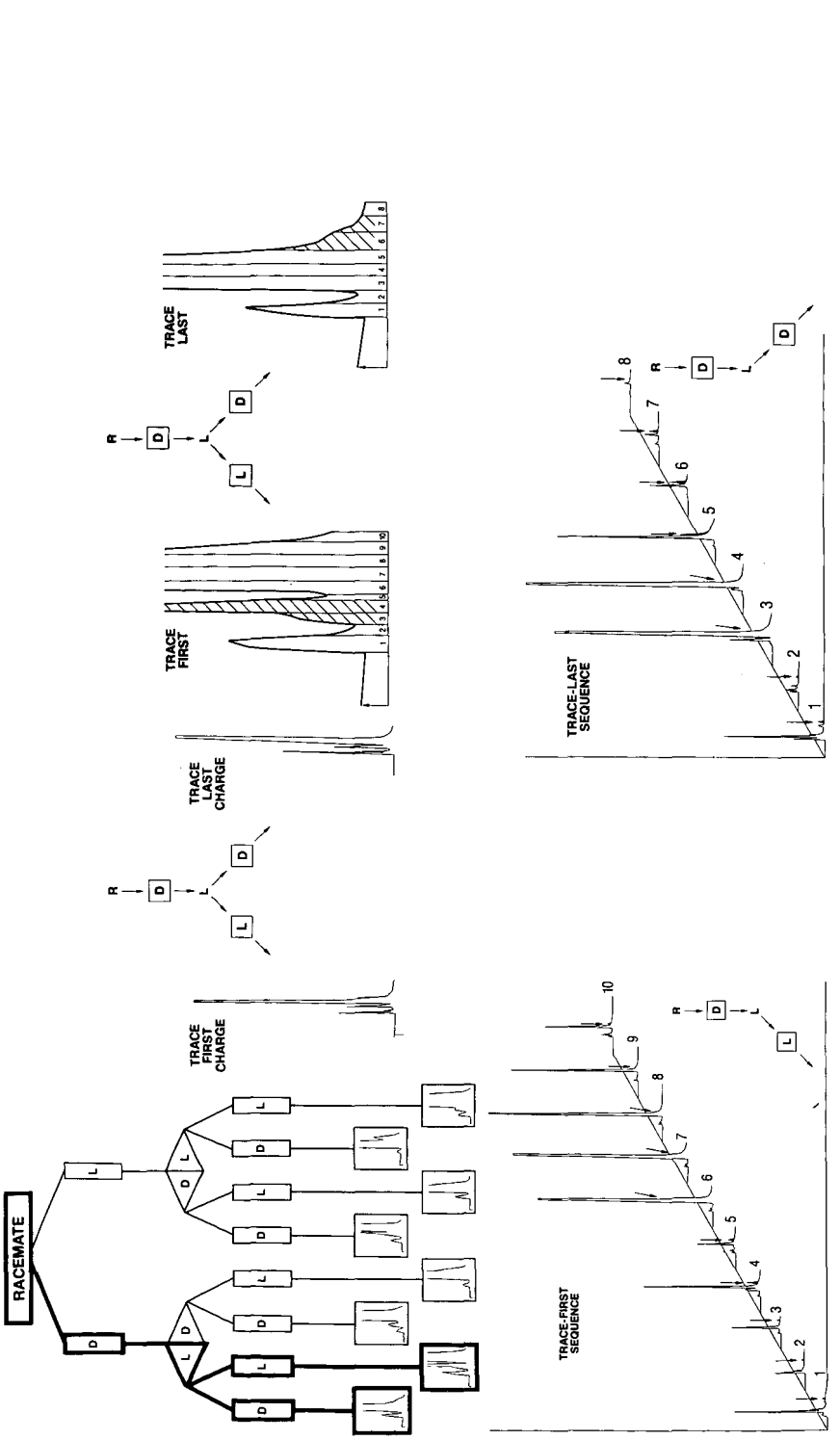



Fig. 1. The diagram at the upper left indicates the pertinent protocol for sample and fraction handling: twice, the racemate was charged to a preparative column that contained the D-enantiomer as CSP, and the L-enriched fractions were separately collected. One L-fraction was then charged to an L-column (giving trace-first elution); the other to a D-column (giving trace-last elution). The analytical chromatograms of the respective charges are shown and identified in the middle of the top row of chromatograms; and the resultant preparative chromatograms, showing the locations and numbering of the fractions, at the right of the top row. Note too the symbolic representations of handling: a boxed D or L indicates a D- or L-column; no box, a D- or L-enriched fraction. The sequences of the analytical chromatograms of the trace-first and trace-last chromatograms are shown and identified in the bottom row. Throughout, the trace peaks are both filled in and marked by an arrow. (In these analytical chromatograms, that the trace peak may have been eluted after the major peak is adventitious, resulting merely from use of a given analytical CSP rather than its enantiomer.)

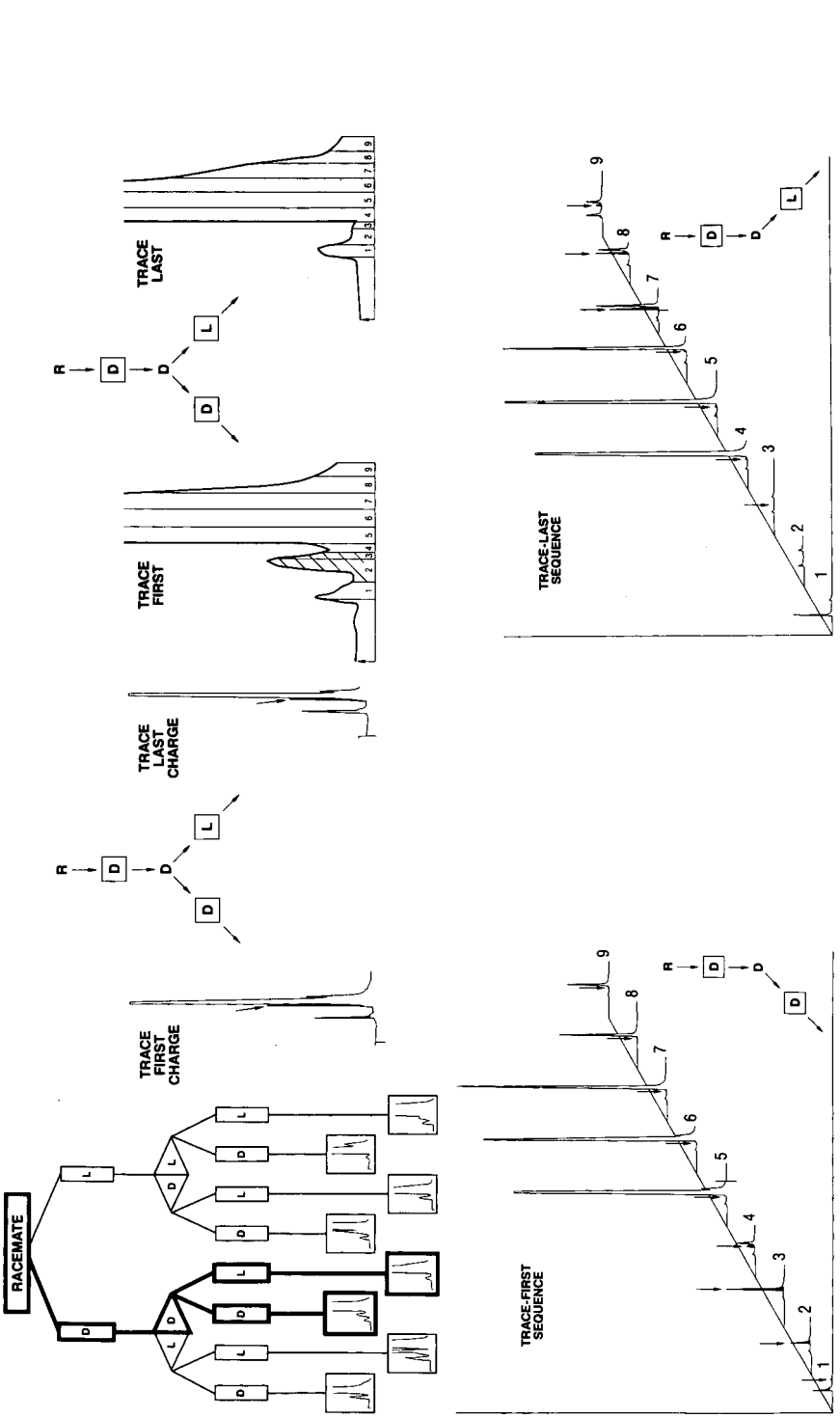



Fig. 2. As in Fig. 1, the diagram at the upper left indicates the pertinent protocol for sample and fraction handling: twice, the racemate was charged to a preparative column that contained the D-enantiomer as CSP. The D-enriched fractions were collected separately and charged separately, one to a D-column (giving trace-first elution); the other to an L-column (giving trace-last elution). The arrangement and identities of the remaining chromatograms are as indicated in Fig. 1 and by the accompanying symbols.

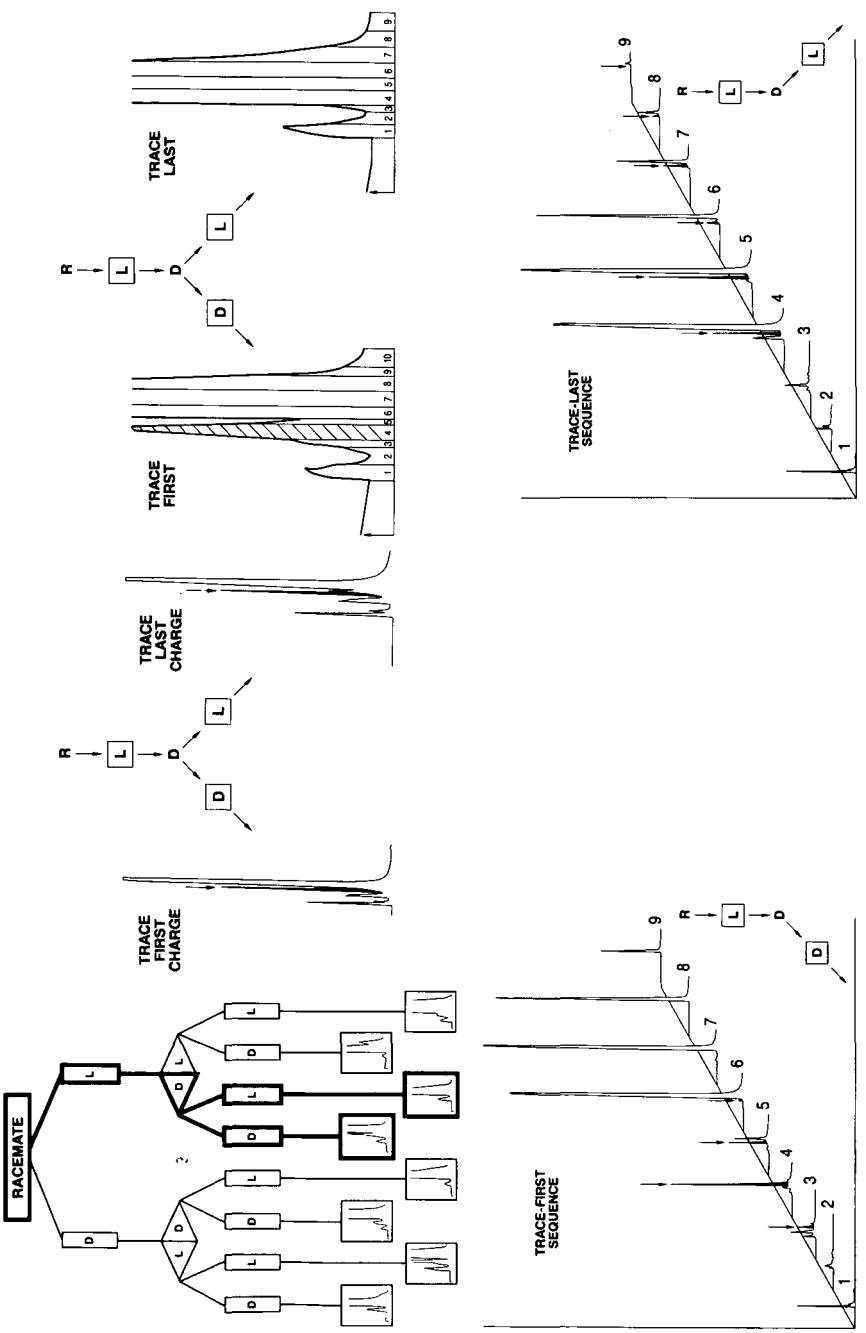



Fig. 3. As in Fig. 1, the diagram at the upper left indicates the pertinent protocol for sample and fraction handling: twice, the racemate was charged to a preparative column that here, however, contained the L-enantiomer as CSP. The D-enriched fractions were collected separately and charged separately, one to a D-column (giving trace-first elution); the other to an L-column (giving trace-last elution). The arrangement and identities of the remaining chromatograms are as indicated in Figs. 1 and 2 and by the accompanying symbols.

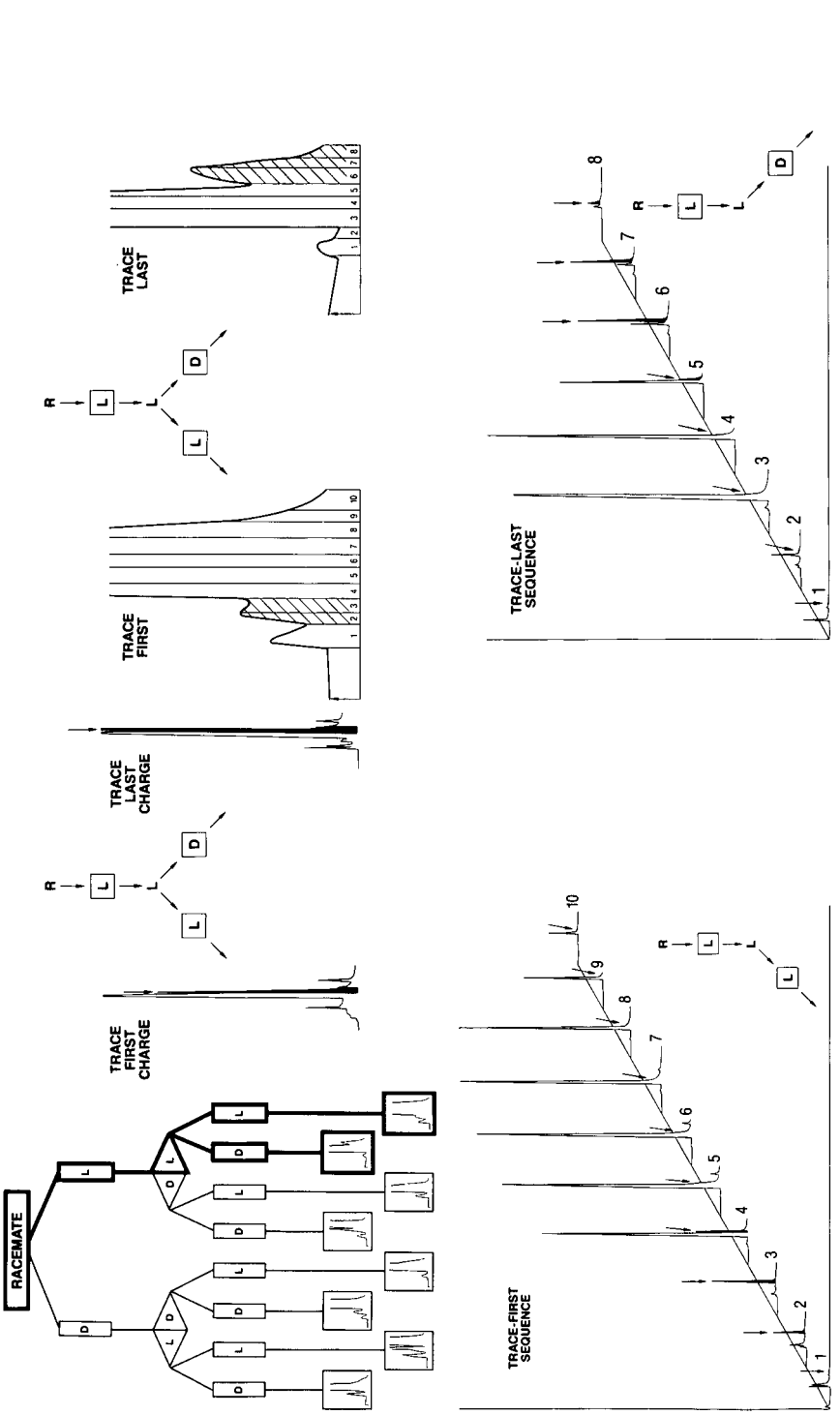



Fig. 4. As in Fig. 3, the diagram at the upper left indicates the pertinent protocol for sample and fraction handling: twice, the racemate was charged to a preparative column that contained the L-enantiomer as CSP. Here, the L-enriched fractions were collected separately and charged separately, one to an L-column (giving trace-first elution); the other to a D-column (giving trace-last elution). The arrangement and identities of the remaining chromatograms are as indicated in Figs. 1, 2 and 3, and by the accompanying symbols.

(Was the Fig. 3, trace-last oscillation of trace concentrations, an artifact? That it was neither an artifact nor merely an unfortunate but at least believable mistake, is suggested by internal evidence within Fig. 3: the trend of major-component concentrations in the successive trace-last chromatograms. In an expectable fashion throughout, these concentrations rise to a maximum in fraction 5 and then diminish gradually.)

Our conclusion from these data was and is that in liquid chromatography, both preparative[8] and high-precision analytical[9], trace components should be eluted before major.

## REFERENCES

1 S. Ghodbane and G. Guiochon, *J. Chromatogr.*, 440 (1988) 9–22.
2 W. H. Pirkle and J. M. Finn, *J. Org. Chem.*, 46 (1981) 2938.
3 W. H. Pirkle and M. H. Hyun, *J. Org. Chem.*, 49 (1984) 3043.
4 W. H. Pirkle and M. H. Hyun, *J. Org. Chem.*, 47 (1985) 287–293.
5 W. H. Pirkle and M. H. Hyun, *J. Chromatogr.*, 328 (1985) 1–9.
6 W. H. Pirkle, G. Mahler and M. H. Hyun, *J. Liq. Chromatogr.*, 9 (1986) 433–453.
7 W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 108 (1986) 352–354.
8 J. A. Perry, J. D. Rateike and T. J. Szczerba, *J. Liq. Chromatogr.*, 9 (1986) 3297–3309.
9 J. A. Perry, J. D. Rateike and T. J. Szczerba, *J. Chromatogr.*, 389 (1987) 57–64.

*Journal of Chromatography*, 503 (1990) 411–419
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 175

# Separation of poly(styrene–vinyl acetate) block copolymers by liquid adsorption chromatography

SADAO MORI
*Department of Industrial Chemistry, Faculty of Engineering, Mie University, Tsu, Mie 514 (Japan)*
(Received October 3rd, 1989)

SUMMARY

Poly(styrene–vinyl acetate) block copolymers, prepared by using a polymeric peroxide as an initiator, were characterized by liquid adsorption chromatography (LAC) and size-exclusion chromatography (SEC). The copolymers were separated according to their chemical composition by LAC using silica gel as an adsorbent and a mixture of 1,2-dichloroethane (DCE) and ethanol as the mobile phase with a 20-min linear gradient from DCE–ethanol (98:2, v/v) to DCE–ethanol (85:15, v/v). The column temperature was 30°C. The copolymers were separated by LAC according to the composition in order of increasing vinyl acetate content. The copolymers were fractionated by LAC and SEC in order to determine the relationship between the composition and molecular weight. The LAC fractions were subjected to SEC and the SEC fractions to LAC. The range of composition for the copolymers was between 40 and 90% (w/w) styrene regardless of the monomer feed. There are positive trends in the composition and the molecular weight averages that the copolymer fractions containing more styrene have lower molecular weights, although the copolymers have chemical compositions and molecular weight distributions that are independent of each other.

## INTRODUCTION

Copolymers in general have a molecular weight distribution and a chemical composition distribution, and the determination of both distributions is very important for the characterization of the copolymers. The separation of poly(styrene–methyl methacrylate) random copolymers[1–5], P(S–MMA), and the block copolymers[6], P(S–b–MMA), according to chemical composition by liquid adsorption chromatography (LAC) has been reported in the previous papers. For the random copolymers, those having more styrene eluted first and the molecular weight dependence on retention volume was not observed[2,3]. Although the separation of the block copolymers was not directly proportional to the composition, LAC was found to be a useful technique for the characterization of the block copolymers.

0021-9673/90/$03.50 

Several attempts have been reported for the separation of random copolymers such as P(S–MMA)[7] and poly(styrene–acrylonitrile)[8] copolymers according to chemical composition by high-performance liquid chromatography (HPLC). However, the reports on the determination of the chemical heterogeneity of block copolymers by HPLC are limited and only one type of styrene block copolymer could be found in the literature apart from our report, *i.e.*, P(S–MMA) block copolymers were separated by LAC and characterized by light scattering[9].

In this work, styrene–vinyl acetate block copolymers, P(S–b–VAc), were characterized by LAC and size-exclusion chromatography (SEC). The separation was in order of increasing vinyl acetate content and molecular weight.

## EXPERIMENTAL

### *Apparatus*

A Model Trirotar-VI high-performance liquid chromatograph (Japan Spectroscopic, Tokyo, Japan) was used for both LAC and SEC. A Model Uvidec-VI UV absorption detector (Japan Spectroscopic) was used for LAC and a Model SE-61 differential refractive index detector (Showa Denko, Tokyo, Japan) for SEC. The UV detector was operated at 254 nm. The column used for LAC (50 mm × 4.6 mm I.D.) was packed with silica gel of pore diameter 30 Å and particle size 5 μm (Nomura Chemical, Aichi, Japan). The column was thermostated at a specified temperature in a Model TU-300 column oven (Japan Spectroscopic).

SEC columns were two Shodex A80M HPSEC columns (50 cm × 8 mm I.D.) (Showa Denko) packed with polystyrene gel for polymer separation.

### *Samples*

The samples used were styrene–vinyl acetate block copolymers, P(S–b–VAc), prepared at the Chemicals & Explosives Laboratory, Nippon Oil & Fats, Aichi, Japan). Vinyl acetate monomer was first polymerized with a polymeric peroxide as the initiator, then styrene monomers were added and the polymerization process was continued. Three samples of P(S–b–VAc) of different compositions were prepared by changing the monomer concentrations; the styrene feed for P(S–b–VAc) I was 90 wt.-%, that for P(S–b–VAc) II was 70 wt.-% and that for P(S–b–VAc) III was 50 wt.-%.

The products were subjected to Soxhlet extraction, first with cyclohexane to remove polystyrene (PS), then with methanol to remove poly(vinyl acetate) (PVAc). The residues consisted of P(S–b–VAc) as the main product and small amounts of residual PS and PVAc homopolymers. The composition of the purified products was measured by NMR spectroscopy. The styrene contents of the copolymers were I, 90 wt.-%; II, 48 wt.-%; and III, 49 wt.-%.

### *Elution*

The mobile phase for LAC was a mixture of 1,2-dichloroethane (DCE) and ethanol. The composition of the mobile phase was regulated by linear gradient elution. The initial mobile phase (A) was DCE–ethanol (98:2, v/v), the composition of the final mobile phase (B) was DCE–ethanol (85:15, v/v) and the composition of the mobile phase was changed from 100% A to 100% B in 20 min linearly. The copolymer samples

were dissolved in DCE at a concentration of 0.1% and the sample solutions were injected onto the column 1 min after from the start of the gradient elution. The column temperature was 30°C. The flow-rate of the mobile phase was 0.5 ml/min and the injection volume of the sample solutions was 0.1 ml. Isocratic elution was also performed for the selection of the elution conditions by changing the composition of the mobile phase and the column temperature.

The mobile phase for SEC was tetrahydrofuran (THF) at a flow-rate of 1 ml/min. The copolymer samples were dissolved in THF at a concentration of 0.1% and the injection volume was 0.2 ml.

*Fractionation*

In order to estimate the chemical heterogeneity of the block copolymers, the LAC traces were divided into ten fractions as shown in Fig. 2 by dividing the retention volume every 0.5 ml from 4.5 to 9.5 ml. The sample concentration was 0.4% and the injection volume was 0.2 ml. Fractionation was repeated 15 times and corresponding fractions were combined. One portion of the fractions was used for the determination of composition and the other for the determination of molecular weight averages.

SEC traces for the copolymers were divided into six fractions as shown in Fig. 3 by dividing the retention volume every 2 ml from 21 to 33 ml. Fractionation was repeated five times and corresponding fractions were combined. The mobile phase was DCE–ethanol (95:5, v/v); the sample concentration was 0.4% and the injection volume was 0.2 ml. Solvent in the fractions was removed and the residues were dissolved in DCE.

## RESULTS AND DISCUSSION

*Selection of eluton conditions*

The reactivity ratios between the two monomers, styrene ($M_1$) and vinyl acetate ($M_2$), are $r_1 = 55 \pm 10$ and $r_2 = 0.01 \pm 0.01$. These two values are significantly different and hence a styrene–vinyl acetate random copolymer cannot be obtained by usual polymerization techniques. Therefore, in this study, the block copolymers were used as the test samples to select the elution conditions.

At a column temperature of 30°C, the elution behaviour of the copolymers was examined by changing the ethanol content in DCE as the mobile phase. Isocratic elution was performed. When DCE and mixtures of DCE and ethanol up to 98:2 (v/v) were used as the mobile phase, all the copolymers were retained in the column, excect for a small peak, which was assigned to styrene, at the interstitial volume of the column system. At a concentration of 3% (v/v) ethanol in the mobile phase, the peak height at the interstitial volume (about 0.5 ml) increased to some extent, and it increased with increasing ethanol content in the mobile phase. However, no peaks were observed after the interstitial volume. This observation was similar to that observed for styrene–methyl methacrylate copolymers[2], where all the block copolymers and polystyrene appeared at the interstitial volume on increasing the ethanol content in the mobile phase.

Linear gradient elution was then attempted for eluting the copolymers at different retention volumes according to their chemical compositions, with a 20-min gradient from DCE–ethanol (98:2, v/v) to DCE–ethanol (85:15, v/v). All the

copolymers and PVAc were retained in the column and only PS could be eluted from the column with the initial mobile phase. With increase in the ethanol content, the copolymers appeared from the column in order of increasing vinyl acetate content. The results are shown in Fig. 1. DCE alone was also injected as a sample solution to check the baseline drift.

The first peak, appearing at $V_R$ = 0.5–1.0 ml, was residual PS wich remained in the samples despite the extraction process. The second peak was UV-sensitive impurities produced during polymerization. Most of the impurities were removed by the purification process by dissolving the copolymers in chloroform, followed by precipitation in methanol. The peak at $V_R$ = 2.5 ml and the breakthrough at $V_R$ = 4.0–4.5 ml were impurities or the baseline drift originated in the DCE solvent, as shown in Fig. 1 D. The block copolymers appeared between 4.7 and 9.3 ml. Only one broad and leading peak was observed for every sample. The chromatograms were similar for the three samples. A PVAc homopolymer must appear at the end of the chromatogram, *i.e.*, around 9.0 ml, although it cannot be observed with a UV detector at 254 nm.

LAC traces for the three samples were also obtained at different column temperatures. Similar chromatograms to those in Fig. 1 were obtained, but the retention volumes of the peaks for the copolymers decreased with increasing column temperature. A PVAc homopolymer does not have UV absorption at 254 nm, and therefore these chromatograms represented styrene only.

*Fractionation by LAC*

In order to determine the compositions and molecular weights of the block copolymers appearing at different retention volumes in the chromatograms in Fig. 1, the copolymers were fractionated by LAC into nine or ten fractions. LAC results for the copolymers are shown in Fig. 2. The sample load was eight times larger than that in

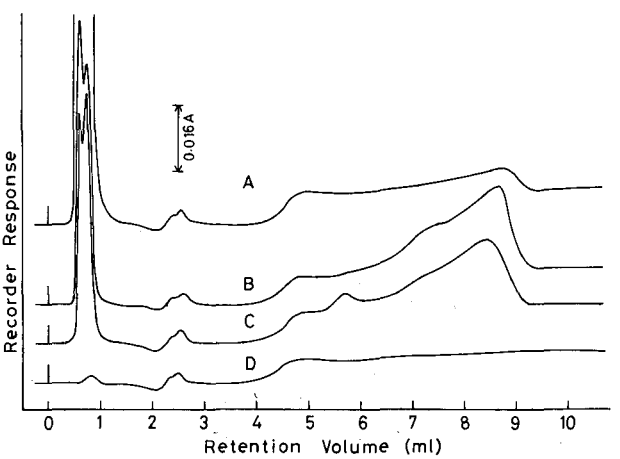



Fig. 1. LAC results for P(S–b–VAc) copolymers obtained with linear gradient elution. Samples: (A) P(S–b–VAc) I; (B) P(S–b–VAc) II; (C) P(S–b–VAc) III; (D) DCE. Sample size, 0.1%, 0.1 ml injected; linear gradient from DCE–ethanol (98:2) to DCE–ethanol (85:15) in 20 min; UV detection at 254 nm, 0.16 a.u.f.s.; column temperature, 30°C.

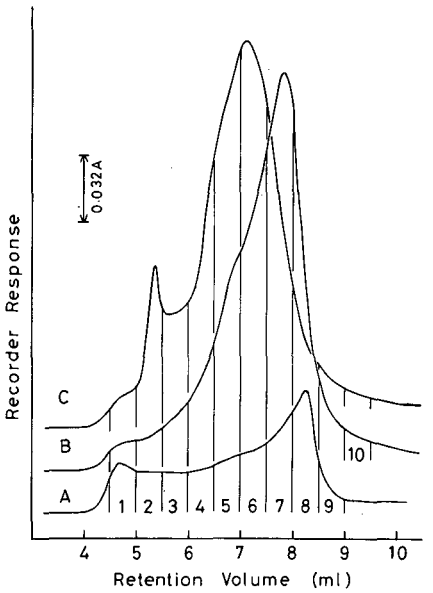



Fig. 2. LAC results and points for fractionation of P(S–b–VAc) copolymers. Samples: (A) P(S–b–VAc) I; (B) P(S–b–VAc) II; (C) P(S–b–VAc) III. Sample size, 0.4%, 0.2 ml injected; UV detection at 254 nm, 0.32 a.u.f.s.; other conditions as in Fig. 1.

Fig. 1 and, as a result, the peak retention volume and the breakthrough volume for the copolymers decreased.

The composition of the copolymers in LAC fractions was measured by infrared spectroscopy. Fractions were dried on a potassium bromide disk and the casting film formed on the disk was subjected to measurement of infrared spectra. The absorbances at 1601 $cm^{-1}$ (a phenyl characteristic band) and 1739 $cm^{-1}$ (a C=O characteristic band) were determined and the styrene content was calculated as follows:

$$\text{Absorbance at } 1601 \text{ cm}^{-1} = 0.0075C + 0.0027 \quad (1)$$

$$\text{Absorbance at } 1739 \text{ cm}^{-1} = 0.1420C' + 0.00166 \quad (2)$$

$$\text{Styrene content in the copolymer (wt.-\%)} = \frac{C}{C + C'} \cdot 100 \quad (3)$$

where $C$ and $C'$ are the styrene concentration in the solution and the vinyl acetate concentration in the solution, respectively.

Eqns. 1 and 2 were obtained with PS and PVAc solutions of several known concentrations by measuring the absorbance and by the least-squares method. The styrene contents in the LAC fractions are given in Table I. Molecular weight averages of the copolymers in the LAC fractions were measured by SEC and the values are also given in Table I. A calibration graph for SEC was constructed with polystyrene standards, and therefore the molecular weights obtained were all polystyrene equivalent molecular weight averages.

Although the three copolymer fractions of the same fraction number were not

TABLE I

STYRENE CONTENT AND MOLECULAR WEIGHT AVERAGES FOR LAC FRACTIONS

| *Sample* | *Fraction No.* | *Styrene content (wt.-%)* | *Molecular weight* | |
|---|---|---|---|---|
| | | | $\bar{M}_n \times 10^{-5}$ | $\bar{M}_w \times 10^{-5}$ |
| I | 1 | 87.4 | 1.46 | 2.41 |
| | 2 | 86.0 | 1.53 | 2.67 |
| | 3 | 87.2 | 1.75 | 2.68 |
| | 4 | 84.6 | 1.65 | 2.49 |
| | 5 | 82.3 | 1.78 | 2.56 |
| | 6 | 74.1 | 1.92 | 2.89 |
| | 7 | 66.3 | 2.42 | 3.60 |
| | 8 | 44.6 | 3.31 | 5.25 |
| | 9 | 33.8 | 3.70 | 5.69 |
| | Unfractionated | 90.0 | 1.29 | 3.09 |
| II | 1 | 90.2 | – | – |
| | 2 | 90.6 | – | – |
| | 3 | 88.8 | 0.91 | 1.51 |
| | 4 | 85.0 | 1.06 | 2.24 |
| | 5 | 86.3 | 1.37 | 2.22 |
| | 6 | 82.9 | 1.59 | 2.40 |
| | 7 | 74.5 | 2.14 | 3.14 |
| | 8 | 50.8 | 3.37 | 5.52 |
| | 9 | 47.4 | 3.20 | 6.60 |
| | 10 | 45.4 | 3.35 | 6.36 |
| | Unfractionated | 48.0 | 1.68 | 6.66 |
| III | 1 | 86.4 | 0.78 | 1.70 |
| | 2 | 74.7 | 0.83 | 1.79 |
| | 3 | 76.2 | 1.37 | 3.08 |
| | 4 | 80.2 | 1.44 | 2.90 |
| | 5 | 81.8 | 1.64 | 2.93 |
| | 6 | 76.5 | 1.89 | 3.07 |
| | 7 | 58.8 | 2.37 | 3.70 |
| | 8 | 54.5 | 2.60 | 4.83 |
| | 9 | 39.1 | 2.35 | 4.89 |
| | 10 | 39.0 | 2.46 | 4.79 |
| | Unfractionated | 49.0 | 1.75 | 5.10 |

consistent with respect to styrene content and molecular weight averages, there are positive trends in the compositions and molecular weight averages. Thus the vinyl acetate content and molecular weight averages of the copolymers increased with increasing fraction number (retention volume), the composition range for the three copolymers was between 40 and 90 wt.-% styrene regardless of the styrene in the feed and the copolymer fractions that contain more styrene have lower molecular weights.

Both the molecular weight distributions and the chemical composition distributions of these fractions were broad. The results from LAC and SEC for the fractions indicated that for the block copolymers the two types of distribution are independent of each other, although there is a relationship between the compositions and the molecular weight averages.

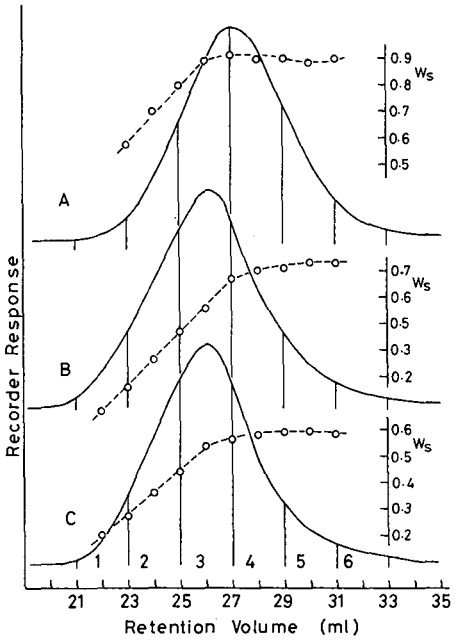



Fig. 3. SEC results and points for fractionation of P(S–b–VAc) copolymers. Samples: (A) P(S–b–VAc) I; (B) P(S–b–VAc) II; (C) P(S–b–VAc) III. Sample size, 0.4%, 0.2 ml injected; UV detection at 254 nm, 0.32 a.u.f.s. ○, Styrene weight fraction, $W_s$. Figures on the chromatogram C represent the fraction numbers.

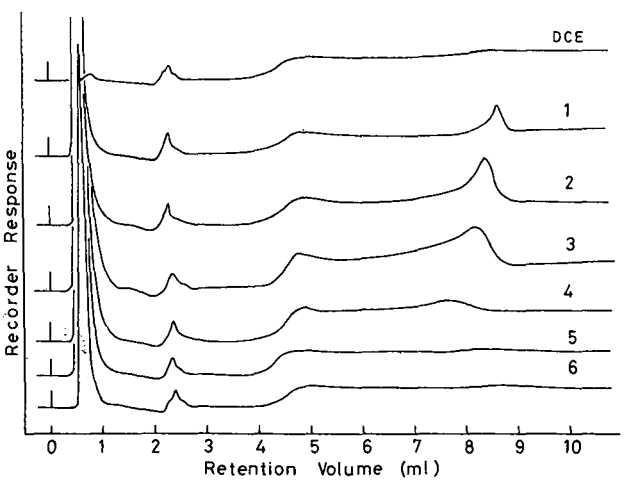



Fig. 4. LAC results for SEC fractions of P(S–b–VAc) I copolymers. Figures on the chromatograms represent the fraction numbers. For LAC conditions, see text.

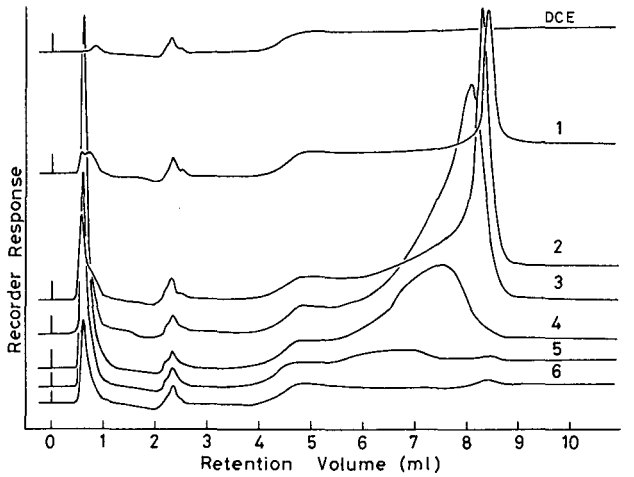



Fig. 5. LAC chromatograms for SEC fractions of P(S–b–VAc) II copolymers. Figures on the chromatograms represent the fraction numbers. For LAC conditions, see text.

*Fractionation by SEC*

In order to discuss in more detail the relationship between the molecular weight and chemical composition distributions, the block copolymers were fractionated by SEC and the fractions were subjected to LAC. SEC results for the copolymers are shown in Fig. 3. Styrene weight fractions calculated point by point were obtained by using a dual detection system (UV and refractive index) and are also shown in Fig. 3. Although the SEC–dual detection system does not give accurate information on the chemical heterogeneity of copolymers[10], the results show the trend in the relationship between composition and molecular weight. Similarly to the results in Table I, the

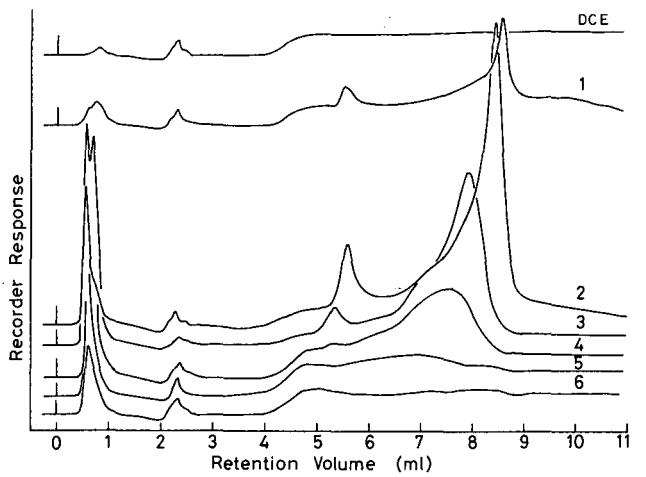



Fig. 6. LAC chromatograms for SEC fractions of P(S–b–VAc) III copolymers. Figures on the chromatograms represent the fraction numbers. For LAC conditions, see text.

portions of the copolymers that contained more styrene eluted later in SEC and had lower molecular weights.

The SEC traces were divided into six fractions and the SEC fractions were subjected to LAC; the results are shown in Figs. 4–6. Fractions having higher molecular weight contain more vinyl acetate. The chemical composition distribution of each fraction was different. Fractions having a higher molecular weight have a narrow chemical composition distribution and fraction 4 had the broadest chemical composition distribution.

A PVAc homopolymer shows UV absorption below 250 nm and the wavelength of maximum absorbance is 237 nm. Therefore, if the wavelength of the UV detector was set at 237 nm, vinyl acetate traces were observed in addition to styrene traces, although the absorption coefficient of PVAc 237 nm was only 22% of that of PS. In this work, however the wavelength at 254 nm was selected in order to obtain a higher sensitivity for the styrene traces.

ACKNOWLEDGEMENTS

The author expresses his gratitude to Mr. H. Ishigaki and Mr. S. Suzuki of Nippon Oil & Fats for sample preparation and to Mr. M. Mouri for technical assistance.

REFERENCES

1 S. Mori, Y. Uno and M. Suzuki, *Anal. Chem.*, 58 (1986) 303.
2 S. Mori and Y. Uno, *Anal. Chem.*, 59 (1987) 90.
3 S. Mori and Y. Uno, *J. Appl. Polym. Sci.*, 34 (1987) 2689.
4 S. Mori, *Anal. Chem.*, 60 (1988) 1125.
5 S. Mori, *Anal. Sci.*, 4 (1988) 365.
6 S. Mori, *J. Appl. Polym. Sci.*, 38 (1989) 95.
7 M. Danielewicz and M. Kubín, *J. Appl. Polym. Sci.*, 26 (1981) 951.
8 G. Glöckner, J. H. M. van den Berg, N. L. J. Meijerink, T. G. Scholte and R. Koningsveld, *Macromolecules*, 17 (1984) 962.
9 T. Tanaka, M. Omoto, N. Donkai and H. Inagaki, *J. Macromol. Sci. Phys.*, 17 (1980) 211.
10 S. Mori, *J. Chromatogr.*, 411 (1987) 355.

*Journal of Chromatography*, 503 (1990) 421–429
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 168

# Determination of N-acetyl- and N-glycolylneuraminic acids in glycoconjugates by reversed-phase high-performance liquid chromatography with ultraviolet detection

NIKOS K. KARAMANOS
*Section of Organic Chemistry, Biochemistry and Natural Products, Department of Chemistry, University of Patras, Patras (Greece)*
BO WIKSTRÖM
*Department of Pathology II, Karolinska Institute, F-42, Huddinge University Hospital, S-141 86 Huddinge (Sweden)*
CHRISTOS A. ANTONOPOULOS
*Section of Organic Chemistry, Biochemistry and Natural Products, Department of Chemistry, University of Patras, Patras (Greece)*
and
ANDERS HJERPE*
*Department of Pathology II, Karolinska Institute, F-42, Huddinge University Hospital, S-141 86 Huddinge (Sweden)*
(First received December 6th, 1988; revised manuscript received November 16th, 1989)

## SUMMARY

A rapid and sensitive method for the determination of sialic acids is described. The measurement is based on isocratic high-performance liquid chromatography, whereby N-acetylneuraminic and N-glycolylneuraminic acids are separated. The total amounts of these acids can be determined after hydrolysis and per-O-benzoylation. The conditions for hydrolysis and derivatization were optimized for measurement of these sialic acids in glycoconjugates.

The benzoyl derivatives were chromatographed on a reversed-phase column with 67% (v/v) aqueous acetonitrile and the eluted peaks were monitored by UV detection. The method allows the determination of picomole amounts. the reaction was shown to give linear calibration graphs over the entire range tested, *i.e.*, up to 160 nmol (50 $\mu$g) of each of the sialic acids.

## INTRODUCTION

Sialic acids are widely distributed in nature, mainly as oligosaccharide components of glycoproteins and gangliosides, but also in glycosaminoglycans[1,2]. They constitute a family of neuraminic acid (5-amino-3,5-dideoxy-D-nonulosonic acid) derivatives. These compounds are divided into two major groups, depending on the

0021-9673/90/$03.50 

nature of their N-substitution. Most naturally occurring forms are N-acetylated, whereas in others the amino group carries a glycolyl group, the relative proportions of these forms differing in various tissues in different species. Often these structures are also acetylated on the hydroxyl groups. Such O-acetylation may be found at one or more of any of the available hydroxyl groups, *i.e.*, at O-4, O-7, O-8 and/or O-9[3,4]. The functions of the different N-acyl and O-acetyl groups are not completely understood, but the variability of these constituents is of importance, *e.g.*, in enzymatic reactions[5,6].

Spectrophotometric[7–13] and enzymatic [14] reactions, thin-layer chromatography[13] and gas chromatography[13,15–17] have all been used to determine sialic acids. Gas–liquid chromatography[18–20] and high-performance liquid chromatography (HPLC)[21] combined with mass spectrometry and also $^1$H NMR spectrometry[22,23] have been applied to the analysis and further elucidation of the structures of more than 30 sialic acids with different acetylation patterns.

Liquid column chromatography, including HPLC, has also been used for the quantification of sialic acids. For such determinations both cation-[24] and anion-exchange[4,12,18,25–29] or anion-exclusion[30] resins have been employed. With such a resin it is possible to separate N-glycolylneuraminic acid (Neu5Gc), N-acetylneuraminic acid (Neu5Ac) and several O-acetylated derivatives of Neu5Ac[6,26]. Reversed-phase resins can also be used to obtain information regarding the content of Neu5Ac[31]. The sensitivity of these methods is limited mainly by the detection mode used. Thus low UV absorbance can be used, but the performance will be improved with postcolumn reactions[30,32] or by the use of precolumn derivatization, when the eluates may be recorded at higher wavelengths. One such method, which involves labelling with a fluorochrome, permits the separation and determination of different sialic acids in small amounts[33].

We have reported[34] that uronic acids and neutral monosaccharides could be determined in picomole amounts by per-O-benzoylation and subsequent HPLC separation. In the same way, hydroxyl groups of sialic acids can be per-O-benzoylated to form stable and strongly UV-absorbing derivatives suitable for the simpler and more accessible UV detection at a higher wavelength.

The liberation of sialic acids from glycoconjugates, which is necessary for their separation and determination, is most often achieved by means of acid hydrolysis[4,12,13,33,35] or enzymatic digestions[6,28]. The latter means of obtaining free sialic acids is dependent on several factors that are difficult to control in biological preparations. Thus O-acetylation, the occurrence of the N-glycolyl group and the conformation of the glycosidic linkage all interfere considerably with the enzymatic activity.

Acid hydrolysis is an effective means of cleaving the glycosidic bonds of the sialic acids. With most acids this liberation is followed by simultaneous removal of O-acetyl groups, whereas the N-acyl linkages are more stable. Most sialic acids can thus be recovered in their two basic forms, Neu5Ac and Neu5Gc. The hydrolytic procedure is always associated, however, with some destruction of these carbohydrates. If such a method is to be used for the release of sialic acids, it is important to check the possible influence on the subsequent derivatization and separation.

This study was therefore undertaken with the aim of finding suitable conditions for the per-O-benzoylation and subsequent chromatographic determination of Neu5Ac and Neu5Gc by HPLC with UV detection. A additional purpose was to find

optimum conditions for the release of these substances from glycoprotein samples, so that the separation procedure could be used for the determination of their contents of Neu5Ac and Neu5Gc.

## EXPERIMENTAL

### *Chemicals*

N-Acetylneuraminic and N-glycolylneuraminic acids and N-acetylneuraminyl-α((2,3)-lactose from human milk were purchased from Sigma (St Louis, MO, U.S.A.). Fetuin (type III) from foetal calf serum, mucin (type I-S) from bovine submaxillary glands and human $\alpha_1$-acid glycoprotein (orosomucoid) were also obtained from Sigma. All other chemicals were of analytical-reagent grade.

### *Sample preparation*

Sialic acids were liberated from glycoproteins by acid hydrolysis. This was performed in 250 μl of 25 m*M* hydrochloric acid or trifluoroacetic acid (TFA) for 2 h at 80°C in screw-capped polypropylene microtubes and the hydrolysates were subsequently lyophilized. As a control of sialic acid destruction, known amounts of Neu5Ac and Neu5Gc were kept under similar hydrolytic conditions. To test the hydrolytic conditions, experiments with various concentrations (up to 100 m*M*) of these acids were also performed. In addition, the effects of 25 m*M* sulphuric acid[33] and 2 *M* acetic acid[35] were tested.

### *Derivatization procedure*

Per-O-benzoylation was performed by a micromodification of the methods described by Daniel *et al.*[36] and Jentoft[31]. To the dry hydrolysate 100 μl of benzoylation mixture [10% (w/v) benzoic anhydride–5% (w/v) *p*-dimethylaminopyridine in pyridine] were added. The mixture was heated at 80°C for 20 min. The conditions for this reaction were also tested by varying the reaction time and temperature. The reaction was terminated by adding 9 volumes of water and shaking vigorously on a vortex mixer. For complete destruction of the remaining benzoic anhydride, the mixture obtained was heated for a further 10 min at 80°C.

Excess of reagents and underbenzoylated derivatives were then removed by passing the mixture through a Sep-Pak $C_{18}$ cartridge (Waters Assoc., Milford, MA, U.S.A.), but eluting directly with 5 ml of water, omitting the aqueous pyridine purification step[36]. The sialic acid derivatives were eluted with only 5 ml of acetonitrile. After evaporation, the dry residue was dissolved in 1 ml of acetonitrile and centrifuged at 10 000 *g* for 5 min in a Beckman Microfuge. Aliquots of up to 20 μl were then injected into the chromatograph. If larger volumes were to be injected, the dried derivatives were preferably dissolved in 67% (v/v) aqueous acetonitrile.

### *High-performance liquid chromatography*

The samples were added to the HPLC column by a loop injector. The separation was performed in a 250 mm × 4.6 mm I.D. column of Supelcosil LC-18 (particle size 5 μm) (Supelco, Bellfonte, PA, U.S.A.), equipped with an RP-18 guard column (30 mm × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.). The samples were eluted with 67% (v/v) aqueous acetonitrile at room temperature and at a flow-

rate of 1.5 ml/min. The eluted peaks were recorded at 231 nm, using a Beckman (Berkeley, CA, U.S.A.) Model 165 variable-wavelength UV detector.

## RESULTS AND DISCUSSION

The per-O-benzoylated derivates of Neu5AC and Neu5Gc gave rise to only one major peak each (Fig. 1). These two peaks were widely separated in the LC-18 column when the samples were eluted with 67% (v/v) aqueous acetonitrile, the retention being 6.9 and 14.8 column volumes (9.2 and 19.5 min), respectively. It is therefore possible to separate these two sialic acids from each other also with higher acetonitrile concentrations, thereby increasing the sensitivity and reducing the elution times. Close to the Neu5AC, however, there are some minor peaks that possibly correspond to underbenzoylated derivatives or perhaps residual benzoic anhydride. In order to avoid coelution of Neu5Ac with these unidentified peaks, it is therefore of advantage to use a mobile phase with higher polarity and consequently longer retention times.

The greater retention of Neu5Gc is compatible with the extra site for benzoylation provided by the glycolyl substituent. Hence it seems as if the Neu5Gc derivative carries one benzoyl group on each of its six hydroxyl residues. This form of sialic acid, however, is more polar than the neutral hexoses because of the presence of the carboxyl and N-glycolyl groups and thus it elutes well before the hexoses. The

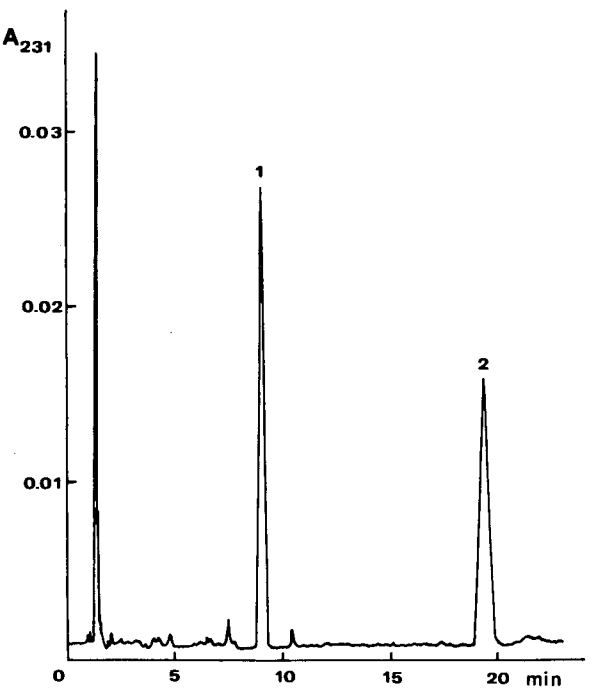



Fig. 1. Chromatogram of the per-O-benzoylated derivatives of (1) Neu5Ac and (2) Neu5Gc. The reversed-phase column (Supelcosil LC-18) was eluted with 67% (v/v) aqueous acetonitrile at 1.5 ml/min and the peaks were recorded at 231 nm.

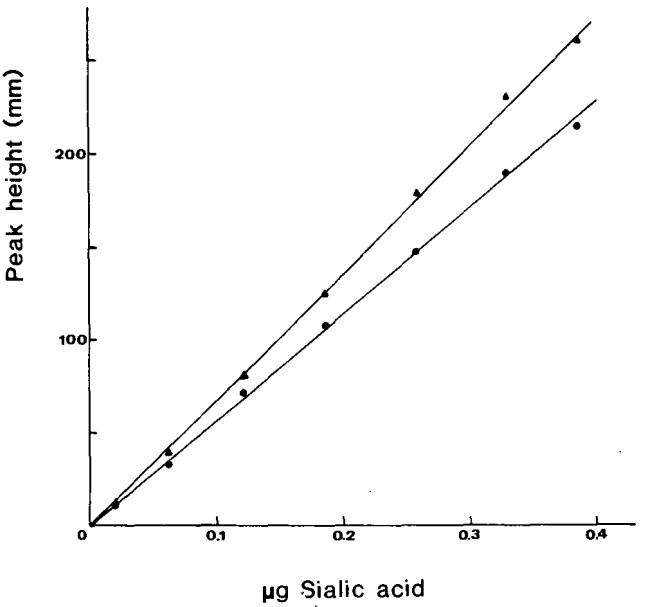



Fig. 2. Calibration graphs obtained with various amounts of (●) Neu5Ac and (▲) Neu5Gc taken for per-O-benzoylation and subsequent chromatography.

retention of Neu5Gc is, in fact, close to that of glucosamine. The anomeric peaks of this hexosamine elute 11.6 and 13.8 column volumes after the front, and will be separated from Neu5Gc with a column performance exceeding 7000 theoretical bottom plates. Galactosamine is recovered in two peaks after 11.6 and 12.3 column volumes, *i.e.*, well before Neu5Gc. When isolated from biological glycoconjugates under the present mild hydrolytic conditions, however, most hexosamines remain N-acetylated and they are therefore eluted much earlier than any of the sialic acids[34].

As with neutral sugars, the per-O-benzoylation will make the carbohydrates highly UV absorbent, allowing their determination in very small amounts. The sensitivity and linearity were both tested with the use of standard sialic acid mixtures (Fig. 2). The reaction with these preparations was linear up to 160 nmol, *i.e.*, when 50 µg of the sialic acid were injected into the column. With this amount the absorbance was close to 2, and it therefore seems as if the range for the analysis is limited mainly by the performance of the detector. The precision of the method was determined by six repeated determinations of both sialic acids. When 15 nmol of each acid were measured, the relative standard deviation was 2.8% for Neu5Ac and 2.6% for Neu5Gc, and with 6 nmol the corresponding figures were 3.1% and 2.9% respectively. The detection limit, expressed as twice the baseline noise, corresponded to 30 pmol (10 ng) of Neu5Ac injected, and as little as 600 pmol (0.2 µg) can thus be determined within a 95% confidence interval. The peak for Neu5Gc was approximately 20% higher than for Neu5Ac, in spite of the much longer retention of the N-glycolyl compound. The differences in peak areas are in good agreement with the assumption that the Neu5Gc derivative contains one more benzoyl residue than Neu5Ac.

With this separation as the measuring device, we studied the conditions for the

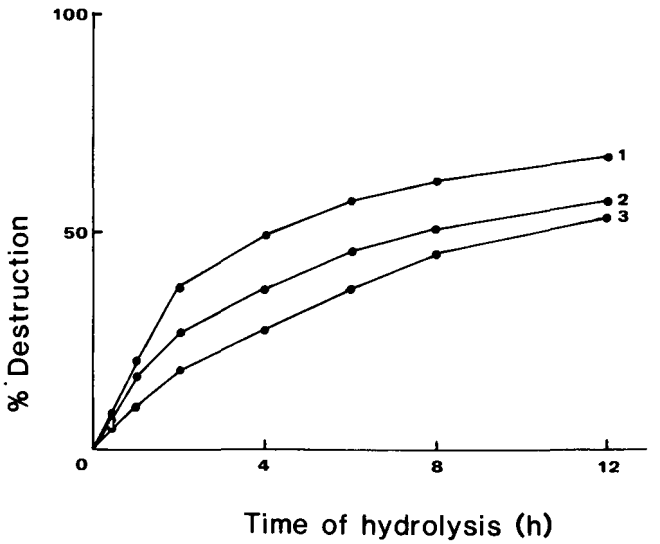



Fig. 3. Destructive effect of the hydrolytic conditions on Neu5Ac with use of (1) 100, (2) 50 and (3) 25 m*M* hydrochloric acid. Similar curves were obtained when the hydrolysis was performed with TFA and also when the effect was tested on Neu5Gc.

derivatization reactions and for the hydrolytic release of sialic acids from glycoconjugates. An increase in temperature from 37 to 80°C during the benzoylation reaction did not significantly affect the reaction yield. At 80°C the rate of the reaction was higher and it was completed within 20 min, whereas at 37°C a reaction time of 90 min was needed. The additional heating of the mixture for 10 min after the termination of the reaction significantly decreased the interference of early eluting peaks, which were also present in blank preparations and thus probably represented the benzoic anhydride.

The effect of the hydrolytic conditions on the Neu5Ac and Neu5Gc standards was studied with various acids as decribed above and at different concentrations. As shown in Fig. 3, there was some destruction depending on the concentration of the acid and the time of hydrolysis. The positions of the eluted peaks were unaltered, and hence there were no signs indicating specific removal of N-acyl groups by any of the acids tested. As shown previously[33], the hydrolysis with 25 m*M* sulphuric acid was associated with only minor losses. These hydrolysates were difficult to lyophilize, however, and it therefore does not seem practical to use this acid. The losses observed with 25 m*M* hydrochloric acid, 25 m*M* TFA and 2 *M* acetic acid were all similar. Acetic acid hydrolysis may, however, leave more O-acetyl groups unhydrolysed, which would then interfere with the subsequent chromatography. Hydrolysis with 25 m*M* hydrochloric acid or TFA seem to be the best of the tested alternatives when studying sialic acids in glycoconjugates. Although hydrolysis with these acids for 2 h will result in losses of *ca.* 20%, this time was preferred to ensure a complete hydrolysis of the glycoconjugate samples (see below).

It has been claimed that the time necessary for the hydrolysis of the sialic acid glycosidic linkages will be dependent on the glycoconjugate[33]. Our results with use of

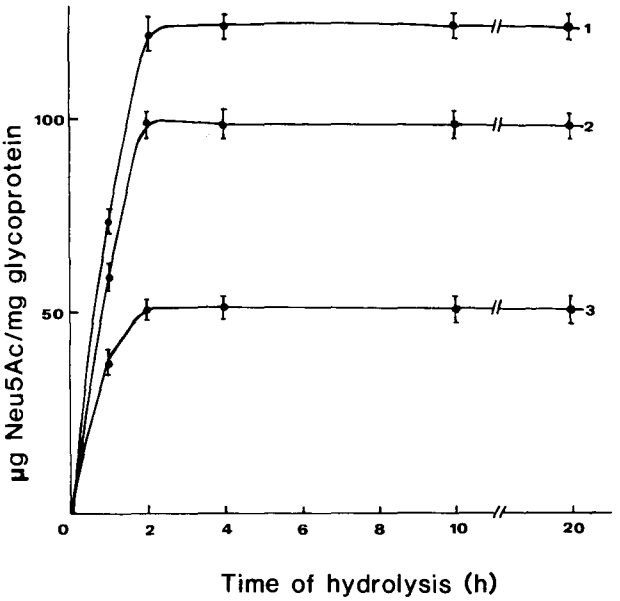



Fig. 4. Rate of liberation of Neu5Ac from glycoproteins when hydrolysis was performed with 25 m*M* hydrochloric acid at 80°C. Similar curves were obtained with 25 m*M* TFA. The amount of liberated sialic acid was determined by comparison with standard sialic acid mixtures treated under the same conditions. 1 = Bovine submaxillary mucin type I-S; 2 = human $\alpha_1$-acid glycoprotein; 3 = foetal calf serum fetuin.

the same commercially available glycoproteins did not verify this. The hydrolytic release of sialic acids reached final levels within 2 h at 80°C, irrespective of the glycoprotein hydrolysed (Fig. 4; *cf.*, ref. 33). The two sialic acid peaks, corresponding to Neu5Ac and Neu5Gc, are readily identified, and are well separated from the elution front. When the glycoprotein contents of these two sialic acids were determined with

TABLE I

SIALIC ACID COMPOSITION OF VARIOUS SAMPLES

Comparison of the present method with values given in the literature.

| *Sample* | *Neu5Ac (%, w/w)* | *Neu5Gc (%, w/w)* | *Total amounts determined spectrophotometrically* | *Ref.* |
|---|---|---|---|---|
| Bovine submaxillary mucin type I-S | 13.0 (11.6–13)[a] | 7.9 (7.4)[a] | 20.9 (22)[a] | 12, 33 |
| Human $\alpha_1$-acid glycoprotein | 10.6 (10.4)[a] | ND[b] ND[b] | (10.8–14.7)[a] | 12, 33, 37 |
| Neu5Ac-α(2,3)-lactose | 46.5 (47.2)[a] | – – | – | 31 |

[a] Values in parentheses were obtained from the literature.
[b] ND = not detected.

the present assay, the values obtained were essentially in agreement with those reported earlier (Table I).

The possibility that some O-acetyl groups may remain after this hydrolysis may also be considered. Such derivatives would then elute earlier than the corresponding sialic acid without the O-acetyl group. In fact, such extra peaks could be observed with considerably shorter times of hydrolysis. With the currently studied glycoproteins, however, no such peaks remained after the 2-h hydrolysis. The presence of detectable amounts of such O-acetylated derivatives from the studied preparations is also contradicted by the hydrolysis curve obtained (Fig. 4). In preparations where the possibility of remaining O-acetyl groups still must be considered, the problem may well be overcome by using a complementary alkaline hydrolysis.

It might seem advantageous to use neuramidase digestion as an alternative to acid hydrolysis, thereby avoiding problems with degradation. This is possible if the digestion is preceded by alkaline hydrolysis releasing O-acetate groups without affecting the sialic acids. Residues from the digestion buffer will, however, interfere with the per-O-benzoylation reaction. The use of neuramidase digests containing the normally recommended buffer would therefore necessitate a tedious purification of the released sialic acids or of the remaining glycoconjugate.

ACKNOWLEDGEMENTS

The authors are indebted to Ms. Mervi Nurminen for skillful technical assistance and to Drs. A. Aletras, T. Tseginidis and D. H. Vynios for helpful discussions. This work was supported by grants from the Swedish Medical Research Council (project no. 8274) and by funds from the Karolinska Institute.

REFERENCES

1 A. P. Corfield and R. Schauer, in R. Schauer (Editor), *Sialic Acids: Chemistry, Metabolism and Function*, Vol. 10, Springer, New York, 1982, pp. 5–50.
2 D. Heinegård and M. Paulsson, in K. Piez and H. A. Reddi (Editors), *Connective Tissue Biochemistry*, Elsevier, New York, 1984, pp. 277–328.
3 A. Rosenberg and C. Schengrund (Editors), *Biological Roles of Sialic Acids*, Plenum Press, New York, 1976.
4 R. Schauer, *Adv. Carbohydr. Chem. Biochem.*, 110 (1982) 131–234.
5 A. P. Corfield, J. C. Michalski and R. Schauer, in G. Tettamanti, P. Durand and S. Di Donato (Editors), *Sialidases and Sialidoses, Perspectives in Inherited Metabolic Diseases*, Vol. 4, Edi Ermes, Milan, 1981, pp. 3–70.
6 A. K. Shukla and R. Schauer, *Anal. Biochem.*, 158 (1986) 158–164.
7 L. Svennerholm, *Acta Chem. Scand.*, 12 (1958) 547–554.
8 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
9 D. Aminoff, *Virology*, 7 (1959) 355–357.
10 D. Aminoff, *Biochem. J.*, 81 (1961) 384–391.
11 G. W. Jourdian, L. Dean and S. Roseman, *J. Biol. Chem.*, 246 (1971) 430–435.
12 M. J. Krantz and Y. C. Lee, *Anal Biochem.*, 63 (1975) 464–469.
13 R. Schauer, *Methods Enzymol.* 50, (1978) 64–89.
14 P. Brunetti, G. W. Jourdian, and S. Roseman, *J. Biol. Chem.*, 237 (1962) 2447–2453.
15 D. A. Graven and C. W. Gehrke, *J. Chromatogr.*, 37 (1986) 414–421.
16 J. Casals-Stenzel, H.-P. Buscher and R. Schauer, *Anal. Biochem.*, 65 (1975) 507–524.
17 Y. Sugawara, M. Iwamori, J. Portoukalian and Y. Nagai, *Anal. Biochem.*, 132 (1983) 147–151.
18 I. Mononen and J. Kärkkäinen, *FEBS Lett.*, 59 (1975) 190–193.

19 J. P. Kamerling, J. Haverkamp, J. F. G. Vliegenthart, C. Verlins and R. Schauer, in A. Frigerio (Editor), *Recent Developments in Mass Spectrometry in Biochemistry and Medicine,* Vol. 1, Plenum Press, New York, 1978, pp. 503–520.
20 R. Schauer, C. Schröder and A. K. Shulka, in R. W. Ledeen, R. K. Yu, M. M. Rapport and K. Suzuki (Editors), *Ganglioside Structure, Function and Biomedical Potential*, Plenum Press, New York, 1984, pp. 75–86.
21 A. K. Shulka, R. Schauer, U. Schade, H. Moll and E. T. Reitschel, *J. Chromatogr.*, 337 (1985) 231–238.
22 J. F. G. Vliegenthart, L. Dorland and H. van Halbeen, in R. Schauer (Editor), *Sialic Acid Chemistry, Metabolism and Function*, Springer, Vienna, 1982, pp. 127–172.
23 J. Haverkamp, H. van Halbeek, L. Dorland, J. F. G. Vliegenthart, R. Pfeil and R. Schauer, *Eur. J. Biochem.*, 122 (1982) 305–311.
24 H. K. B. Silver, K. A. Karim, M. J. Gray and F. A. Salinas, *J. Chromatogr.*, 224 (1981) 381–388.
25 A. K. Shulka, N. Scholz, E. H. Reimerdes and R. Schauer, *Anal. Biochem.*, 123 (1982) 78–82.
26 A. K. Shulka and R. Schauer, *J. Chromatogr.*, 244 (1982) 81–89.
27 S. L. Lohmander, *Anal. Biochem.*, 154 (1986) 75–84.
28 J. R. Scocca, *Anal. Biochem.*, 156 (1986) 61–66.
29 M. L. E. Bergh, P. Koppen and D. H. Van der Eijnden, *Carbohydr. Res.*, 94 (1981) 255–259.
30 S. Honda, S. Iwase, S. Suzuki and K. Kadehi, *Anal. Biochem.*, 160 (1987) 455–461.
31 N. Jentoft, *Anal. Biochem.*, 148 (1985) 424–433.
32 L. D. Powell and G. W. Hart, *Anal. Biochem.*, 157 (1986) 179–185.
33 S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura and Y. Ohkura, *Anal. Biochem.*, 164 (1987) 138–145.
34 N. K. Karamanos, A. Hjerpe, T. Tseginidis, B. Engfeldt and C. A. Antonopoulos, *Anal. Biochem.*, 172 (1988) 410–419.
35 A. Varki, *Anal. Biochem.*, 137 (1984) 236–247.
36 P. F. Daniel, D. F. Feudis, I. T. Lott and R. H. McCluer, *Carbohydr. Res.*, 97 (1981) 161–180.
37 R. W. Jeanloz, in A. Gottschalk (Editor), *Glycoproteins*, Elsevier, Amsterdam, 1972, p. 565.

*Journal of Chromatography*, 503 (1990) 431–436
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 177

# Determination of ascorbic acid, dehydroascorbic acid and ascorbic acid-2-phosphate in infiltrated apple and potato tissue by high-performance liquid chromatography

G. M. SAPERS*, F. W. DOUGLAS, Jr., M. A. ZIOLKOWSKI, R. L. MILLER and K. B. HICKS
*Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, PA 19118 (U.S.A.)*
(Received September 19th, 1989)

## SUMMARY

A high-performance liquid chromatography procedure for the determination of ascorbic acid-2-phosphate (AAP), ascorbic acid (AA), and dehydroascorbic acid (DHAA) in raw apple and potato, treated with AAP and AA to prevent browning, was developed. These compounds were extracted with a mixture of mobile phase and 2.5% metaphosphoric acid and separated on an aminopropyl bonded-phase silica column. DHAA was determined as AA following reduction with dithiothreitol. The method was evaluated with spiked samples and found to be accurate and reproducible at concentrations as high as 0.9 m*M* AA or AAP.

## INTRODUCTION

Recent studies of the control of enzymatic browning in fruits and vegetables have shown that ascorbic acid-2-phosphate (AAP), a stabilized source of ascorbic acid (AA), is a highly effective inhibitor of enzymatic browning in apple tissue[1] and may be applicable to potatoes and other commodities as well. Since this compound must undergo hydrolysis to AA *in situ* to be effective[2], and also since AA functions as a browning inhibitor, at least in part, by undergoing oxidation to dehydroascorbic acid (DHAA) in the course of reducing quinones to polyphenols[3], there is a need to measure all three species in treated samples to evaluate treatment effectiveness. Titrimetric methods, commonly used to determine AA in fruits and vegetables[4], are not sufficiently sensitive to measure residual AA concentrations in treated samples and do not respond to AAP, which is not a reducing agent. Wang *et al.*[5] described a high-performance liquid chromatography (HPLC) procedure for the determination of AA released from L-ascorbic-2-polyphosphate esters following phosphatase digestion. Doner and Hicks[6] employed an aminopropyl bonded-phase silica HPLC column for the determination of AA, or DHAA following reduction to AA with dithiothreitol (DTT). Our objective in the present study was to adapt this procedure to permit the determination of AAP without phosphatase treatment and to develop

0021-9673/90/$03.50 

suitable extraction, clean-up and DTT reaction conditions for the analysis of apple and potato samples containing AAP, AA and DHAA.

## EXPERIMENTAL

### *Chemicals and reagents*

Reagent-grade chemicals and high-purity solvents were used except when specified otherwise. Aqueous solutions were prepared with glass-distilled water. The mobile phase comprised acetonitrile–0.05 *M* potassium dihydrogenphosphate (75:25). Aqueous solutions of AA (Mallinckrodt), DHAA (Aldrich) or AAP (provided by Professor Paul A. Seib, Kansas State University, Manhattan, KS, U.S.A.) were used as dips for apple and potato samples. These compounds were dissolved in mobile phase or a simulated sample extract comprising acetonitrile–aqueous 1.12% metaphosphoric acid (1:1) when used as standards for HPLC analyses. A 2.5% (w/v) solution of DTT (Sigma) in mobile phase was used for DHAA reduction.

### *HPLC procedure*

The HPLC procedure of Doner and Hicks[6] was followed except that separations were carried out on a 25 × 0.46 cm I.D. Rainin 8-μm Dynamax-60A $NH_2$ column, and elution took place isocratically with mobile phase at 2 ml/min. The HPLC system consisted of a Rheodyne Model 7125 injector with a 20-μl sample loop, a Waters Model 6000A pump, a Waters Model 490 detector operated at 254 nm, and a Hewlett-Packard Model 3390A integrator.

### *Dipping procedure*

Plugs were cut from 4–6 apples or potatoes with an electric cork borer, by using a 22-mm I.D. stainless-steel cutting tube, as described previously[7]. The plugs were submerged in aqueous solutions containing 56.8 m*M* AA, AAP or DHAA for 90 s and then drained in a plastic collander for about 30 s. The bottom of the collander was blotted carefully with paper toweling to remove adhering solution. Treated plugs were stored in a dry collander and covered with foil to minimize dehydration.

### *Sample extraction and clean-up*

Plug samples weighing about 30 g were blended with 30 ml aqueous 2.5% metaphosphoric acid solution and 60 ml mobile phase for 2 min at high speed in a Waring Blendor jar. The homogenate was mixed with 2.4 g Celite analytical filter aid (Fisher) and filtered through Whatman No. 541 paper with suction. An additional 2.4-g portion of filter aid was mixed with the filtrate which was then refiltered through Whatman No. 50 paper with suction. Immediately prior to injection, aliquots of the second filtrate were cleaned up by passage through a $C_{18}$ Sep-Pak cartridge (Waters), previously conditioned by flushing with acetonitrile, and an 0.45-μm nylon 66 membrane filter in a 13-mm plastic filter holder (Rainin). Cartridges could be re-used for as many as 12 samples with careful flushing between samples and overnight conditioning in acetonitrile. Samples to be analyzed for total AA were pretreated with DTT to reduce DHAA prior to clean-up. The pH of 25-ml aliquots of second filtrate was adjusted to 6 with 0.3 ml 10% sodium hydroxide, 1.0 ml DTT solution was added with stirring, and the reaction mixture was held at room temperature (*ca.* 20°C) for 30 min prior to clean-up and injection.

*Linearity and recovery experiments*

Standard curves were run for solutions of AA or AAP in simulated sample extract at concentrations between 0.114 and 1.14 m*M* to determine the linear range of the procedure. Initial recovery experiments were carried out by adding aliquots of AA or DHAA solution to blender jars prior to the extraction of apple or potato samples. The recovery of AA and AAP from various apple cultivars and Russet potatoes was determined in replicated trials by spiking freshly prepared extracts with these compounds at concentrations of 0.28, 0.57, and 0.85 m*M* and comparing the HPLC peak areas with those obtained for unspiked samples and solutions of AA or AAP in simulated sample extract. The AA content of spiked samples was corrected for endogenous AA prior to the recovery calculation.

## RESULTS AND DISCUSSION

*HPLC separation of AA and AAP*

Typical chromatograms for the separation of AA and AAP on the aminopropyl column are shown in Fig. 1. No interfering peaks were seen on chromatograms for unspiked apple or potato samples (other than endogenous AA). The retention time of AAP could be reduced by gradient elution, increasing the proportion of buffer to 50%. However, isocratic separation was found to be satisfactory within the time frame of the experiments conducted in this study.

*Linearity of standard curves*

Standard curves for AA and AAP in simulated sample extract in the concentration range 0.114–1.14 m*M* are shown in Fig. 2. A linear relationship between peak area and concentration was obtained at concentrations as high as 0.9 m*M*. Slopes of standard curves were similar for AA and AAP and were consistent from trial to trial.

*Recovery of AA, AAP and DHAA*

Initial studies of AA recovery from Granny Smith apple plugs, spiked with 333 mg/kg AA, yielded values in excess of 95%. Recovery experiments carried out with

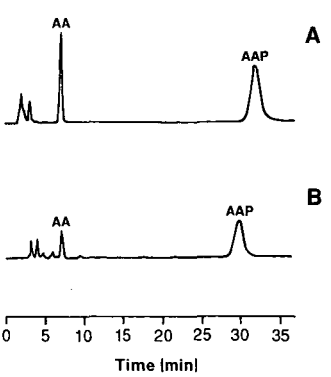



Fig. 1. Chromatograms showing the separation of ascorbic acid (AA) and ascorbic acid-2-phosphate (AAP) on an aminopropyl bonded-phase silica column. (A) Granny Smith apple plugs, 2.5 h after dipping; and (B) Russet Burbank potato plugs, immediately after dipping, each dipped in 56.8 m*M* AAP for 90 s.

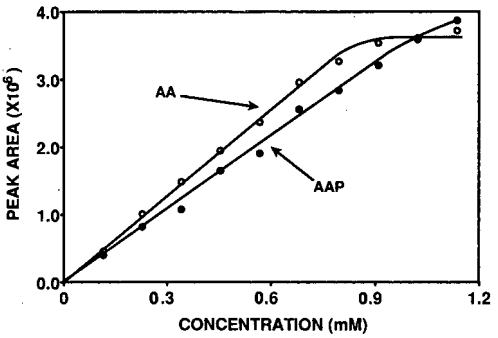



Fig. 2. Standard curves for ascorbic acid (AA) and ascorbic acid-2-phosphate (AAP) in acetonitrile–1.12% metaphosphoric acid (1:1).

spiked apple and potato extracts demonstrated near quantitative recovery of both AA and AAP at concentrations of 0.28, 0.57 and 0.85 m*M* (Table I). The slopes of concentration *vs.* peak area curves were similar for standards and spiked extracts and slightly lower for AA, compared to AAP. Thus, the accuracy of the determination appears to be independent of commodity and AA or AAP concentration, within the range likely to be encountered in treated samples.

DHAA recovery was at least 92% in spiked apple and potato samples (Table II). Neutralization of metaphosphoric acid in sample extracts is essential if quantitative recovery of DHAA is to be obtained. DHAA recovery was only 14% in unneutralized extract (pH 2.9) and 45% at pH 4. These results indicate that by analyzing samples, both with and without DTT reduction, one can follow the oxidation of AA to DHAA due to quinone reduction and other oxidation reactions.

TABLE I

RECOVERY OF ASCORBIC ACID (AA) AND ASCORBIC ACID-2-PHOSPHATE (AAP) FROM SPIKED APPLE AND POTATO EXTRACTS

| *Sample* | *Compound* | *Slope*[a] | | *Recovery*[b] *(%)* | | |
|---|---|---|---|---|---|---|
| | | *Standard curve* | *Spiked extracts* | *Level of spiking (mM)* | | |
| | | | | *0.28* | *0.57* | *0.85* |
| Granny Smith apple | AA | 4.30 | 4.08 | 102 | 95 | 95 |
| | AAP | 5.10 | 4.99 | 100 | 103 | 97 |
| Red Delicious apple | AA | 3.97 | 4.26 | 97 | 121 | 104 |
| | AAP | 4.99 | 5.07 | 127 | 108 | 109 |
| Golden Delicious apple | AA | 4.00 | 4.27 | 94 | 96 | 100 |
| | AAP | 5.09 | 5.38 | 95 | 100 | 102 |
| Russet potato | AA | 3.97 | 3.87 | 97 | 91 | 98 |
| | AAP | 4.42 | 5.13 | 103 | 116 | 112 |

[a] For peak area ($\times 10^6$) *vs.* concentration (m*M*); correlation coefficients for regression > 0.99.
[b] Based on 2–4 replicate determination for each standard and spiked extract.

TABLE II

RECOVERY OF DEHYDROASCORBIC ACID (DHAA) FROM SPIKED APPLE AND POTATO PLUGS AND SIMULATED EXTRACTS

| *Sample* | *DHAA added*[a] *(μg/ml)* | *Total ascorbic acid (TAA) found* | | |
|---|---|---|---|---|
| | | *Endogenous (μg/ml)* | *Spiked (μg/ml)* | *Recovery*[b] *(%)* |
| Winesap apple, pH 2.9 | 50.6 | 3.3 | 10.5 | 14 |
| Simulated extract, pH 4 | 50.6 | – | 22.8 | 45 |
| Simulated extract, pH 5 | 50.6 | – | 50.2 | 99 |
| Granny Smith apple[c] | 35.7 | 4.9 | 40.2 | 99 |
| Red Delicious apple[c] | 35.7 | 3.3 | 36.3 | 92 |
| Russet potato[c] | 36.6 | 27.3 | 62.9 | 97 |

[a] Calculated as μg ascorbic acid/ml sample extract.

[b] Recovery $= \frac{(\text{TAA spiked} - \text{TAA endogenous})}{\text{DHAA added}} \cdot 100.$

[c] Adjusted to pH 6.

TABLE III

PRECISION OF HPLC DETERMINATION OF ASCORBIC ACID (AA) AND ASCORBIC ACID-2-PHOSPHATE (AAP) IN APPLE AND POTATO PLUGS

| *Sample* | *Treatment* | *Compound determined* | *Peak area* | | *Coefficient of variation* |
|---|---|---|---|---|---|
| | | | *Mean ± S.D.* | *n* | |
| Standard | 0.57 m*M* AA | AA | 953 500 ± 77 360 | 9 | 8.1 |
| Golden Delicious | Spiked with 0.57 m*M* AAP | AAP | 2 735 700 ± 104 800 | 8 | 3.8 |
| Red Delicious | Dipped in 56.8 m*M* | AA | 1 790 100 ± 41 300 | 7 | 2.3 |
| | AAP, held 48 h | AAP | 1 060 800 ± 85 700 | 7 | 8.1 |
| Russet potato | Dipped in 56.8 m*M* | AA | 2 423 600 ± 111 300 | 4 | 4.6 |
| | AAP, held 24 h | AAP | 359 400 ± 51 400 | 4 | 14.3 |

TABLE IV

ASCORBIC ACID-2-PHOSPHATE (AAP), ASCORBIC ACID (AA), AND DEHYDROASCORBIC ACID (DHAA) IN INFILTRATED GRANNY SMITH APPLE PLUGS

Apple plugs infiltrated with 56.8 m*M* AAP solution and stored at *ca.* 20°C.

| *Time after dipping (h)* | *Concentration (μmol/100 g)* | | |
|---|---|---|---|
| | *AAP* | *AA* | *DHAA* |
| 0.1 | 218.5 | 10.5 | 6.1 |
| 5 | 111.5 | 171.0 | 30.9 |
| 24 | 9.7 | 241.5 | 10.4 |
| 48 | 0 | 165.4 | 21.5 |

*Precision of method*

Examples of the precision of the HPLC method are shown in Table III. Typically, coefficients of variation were less than 10%, except when residual concentrations of AA or AAP were small, as in the Russet potato sample. With this degree of precision, the HPLC method is suitable for determinations of AA or AAP uptake in treated samples or for investigations of AAP hydrolysis and subsequent oxidation following treatment.

*Application of HPLC method to infiltrated samples*

Data in Table IV show the application of the HPLC method to Granny Smith apple plugs, infiltrated with AAP by dipping in a 56.8 m*M* solution for 90 s and then stored at *ca.* 20°C for 48 h. It can be seen that the AAP concentration in treated plugs decreased almost to zero within 24 h while the AA concentration increased, presumably due to AAP hydrolysis by endogenous acid phosphatase. The DHAA concentration in the infiltrated plugs was relatively small throughout the storage period. An investigation of the uptake and fate of AAP in infiltrated fruits and vegetables will be reported elsewhere.

AAP might be used as a stable form of Vitamin C in various food or feed products, cosmetics or pharmaceuticals[2]. With minor modification, the HPLC procedure described herein would be applicable to the evaluation of these systems as well as infiltrated fruits and vegetables.

REFERENCES

1 G. M. Sapers, K. B. Hicks, J. G. Phillips, L. Garzarella, D. L. Pondish, R. M. Matulaitis, T. J. McCormack, S. M. Sondey, P. A. Seib and Y. S. El-Atawy, *J. Food Sci.*, 54 (1989) 997.
2 P. A. Seib and M. L. Liao, *U.S. Pat.*, 4 647 672 (1987).
3 L. Vamos-Vigyazo, *CRC Crit. Rev. Food Sci. Nutr.*, 15 (1981) 49.
4 *Methods of Analysis of AOAC*, Association of Official Analytical Chemists, Washington, DC, 14th ed., 1984, method No. 43.064, p. 844.
5 X.-Y. Wang, M.-L. Liao, T.-H. Hung and P. A. Seib, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 1158.
6 L. W. Doner and K. B. Hicks, *Methods Enzymol.*, 122 (1986) 3.
7 G. M. Sapers and F. W. Douglas, Jr., *J. Food Sci.*, 52 (1987) 1258.

*Journal of Chromatography*, 503 (1990) 437–441
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 180

# Note

## Chiral organosilicon compounds

## II[a]. Comparison of gas–liquid chromatographic and NMR analyses of enantiomeric alcohols

PERRY T. KAYE* and ROBIN A. LEARMONTH
*Department of Chemistry and Biochemistry, Rhodes University, P.O. Box 94, Grahamstown 6140 (South Africa)*
(First received August 9th, 1989; revised manuscript received November 28th, 1989)

The determination of enantiomeric excess is fundamental to the evalution of asymmetric induction methodologies and the literature reflects a continuing interest in techniques for enantiomer differentiation. These techniques include gas–liquid chromatographic (GLC) analysis using chiral stationary phases and chiral derivatization agents[2–5] and NMR analyses using chiral solvating agens[6,7], chiral platinum complexes[8] and chiral imidazolidine and boronic acid derivatization agents[9–11]. Whereas the derivatization capabilities of organosilicon compounds are well established, applications involving chiral organosilicon systems in synthesis and analysis have, until recently, received little attention[12,13]. NMR applications involving chiral silyl derivatives appear to be limited to studies by Richter[14], who used $^{1}H$ NMR analysis to examine stereoselectivity in the reactions of prochiral siloxanes, and, more recently, by Chan *et al.*[15], who used NMR ($^{1}H$, $^{13}C$ and $^{29}Si$) methods to differentiate diastereomeric silyl acetal derivatives of enantiomeric alcohols. To our knowledge, no applications of chiral silyl probes in GLC have been reported. In this paper we compare GLC and NMR ($^{1}H$ and $^{13}C$) analyses of diastereomeric silyl acetals (**1–5**, Table I) [including the silyl acetals (**4**) examined by Chan *et al.*[15]], obtained by reacting selected racemic alcohols with excess of borneol- and/or menthol-derived chiral chlorosilanes [prepared from (−)-menthol and (−)-borneol as described in Part I[1]], under conditions which may be expected to minimize any kinetic resolution effects.

### EXPERIMENTAL

The synthesis of the diastereomeric silyl acetals **1–5**, all of which gave satisfactory NMR, IR and high-resolution mass spectrometric analyses, was effected under anhydrous conditions in an inert atmosphere (nitrogen) as illustrated by the

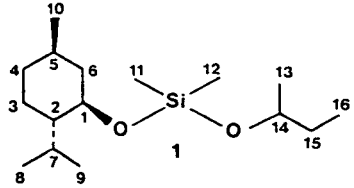
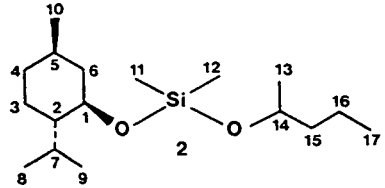
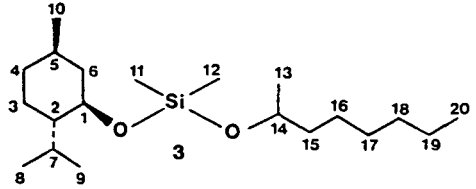
[a] For Part I, see ref. 1.

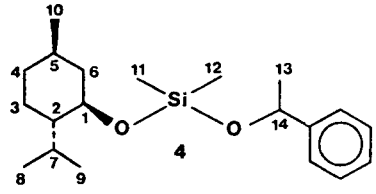
0021-9673/90/$03.50 

TABLE I

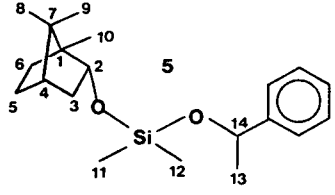
GLC AND NMR DATA FOR DIASTEREOMERIC SILYL ACETALS, $R^1(CH_3)_2SiOCH(R^2)R^3$

$t_R$ = Retention times for 30- and 60-m columns followed, in each instance, by relative peak areas in parentheses. $R_S$ = Peak resolution. $\alpha$ = Separation factor. $\delta_H$ = Chemical shifts followed, in parentheses, by relative signal intensity ratios, signal multiplicities and signal assignments. $\delta_C$ = $^{13}C$ P.n.d. signal shifts followed by signal assignments.

| *Compound* | *$t_R$ (min)* | *$R_S$* | *$\alpha$* | *$\delta_H$ (ppm)* | *$\delta_C$ (ppm)* |
|---|---|---|---|---|---|
|  1 | – | – | – | 1.13, 1.14 (0.99:1.00; 2 × d[a]; 13-H) | 45.39, 45.44; C-6 |
|  2 | – | – | – | 1.13, 1.14 (0.91:1.00; 2 × d[b]; 13-H) | 23.58, 23.66; C-13<br>45.39, 45.44; C-6 |
|  3 | 35.60, 35.78 (0.88:1.00)<br>71.63, 71.84 (0.85:1.00) | 0.69<br>0.75 | 1.005<br>1.003 | 1.13, 1.14 (0.81:1.00; 2 × d[c]; 13-H) | 23.58, 23.66; C-13 |




| | | | | |
|---|---|---|---|---|
| | | | 1.47, 1.48 (1.00:0.98; | 15.92, 15.97; C-9 |
| 41.14, 41.35 (1.00:1.00) | 0.80 | 1.005 | 2 × d[d]; 13-H) | 22.12, 22.23; C-8 |
| 84.37, 84.70 (0.95:1.00) | 0.88 | 1.004 | 1.79, 1.96 (0.98:1.00; | 31.59 31.65; C-5 |
| | | | 2 × m; 6-$H_{eq.}$) | 45.23, 45.37; C-6 |




| | | | | |
|---|---|---|---|---|
| | | | 0.847, 0.851 (0.93:1.00; | 13.34, 13.47; C-10 |
| 43.00, 43.16 (1.00:0.98) | 0.38 | 1.004 | 2 × s; 8-, 9- or 10-H) | 28.25, 28.29; C-5 |
| 88.37, 88.68 (0.92:1.00) | 0.63 | 1.004 | 1.56, 1.59 (0.90:1.00; | 39.13, 39.18; C-3 |
| | | | 2 × t; 4-H) | 45.18, 45.22; C-4 |

[a] $J$ = 6.18 and 6.03 Hz, respectively.
[b] $J$ = 5.94 and 6.15 Hz, respectively.
[c] $J$ = 5.85 and 6.03 Hz, respectively.
[d] $J$ = 6.34 and 6.30 Hz, respectively.

following example. A solution of chloro(menthyloxy)dimethylsilane (2.98 g, 12 mmol)[1] in diethyl ether (10 ml) was added dropwise to a stirred solution of racemic 1-phenylethanol (0.98 g, 8 mmol) (the *racemic* alcohol substrates exhibited zero optical rotation) and triethylamine (1.2 g, 12 mmol) in diethyl ether (20 ml). After stirring overnight, the resulting slurry was poured into aqueous sodium hydrogencarbonate. Extraction with diethyl ether, concentration *in vacuo* and flash chromatography on silica [elution with hexane–ethyl acetate (95:5)] gave, as a diastereomeric mixture, menthyloxydimethyl(1-phenylethoxy)silane (**4**) (1.9 g, 71%). Isolated yields for the other silyl acetals were: (**1**) 72%, (**2**) 76% (**3**) 50% and (**5**) 57%.

The silyl acetals **1**–**5** were chromatographed, as 0.1–0.2% solutions in diethyl ether, on a Hewlett-Packard 5890A gas chromatograph fitted with a J&W DB225 fused-silica capillary column (0.25 $\mu$m film thickness) [30 m × 0.25 mm I.D. (for a 60-m column, two 30-m columns were joined)] and using helium as the carrier gas at a flow-rate of 41 ml/min, optimized temperature programmes [*e.g.*, for the silyl acetals **4**, 60–140°C at 4°C/min, 140–170°C at 1°C/min], an inlet purge time of 0.5 min and flame ionization detection using synthetic air and hydrogen as detector feed gases. The inlet and detector temperatures were set at 210°C.

$^1H$ (500 MHz) and $^{13}C$ (125 MHz) NMR spectra were obtained for $C^2HCl_3$ solutions on a Bruker WM 500 spectrometer.

## RESULTS AND DISCUSSION

Silyl acetal mixtures (**1**–**5**) were chromatographed on a standard 30-m capillary column. Under optimum temperature-programmed conditions, resolution of the components (see Fig. 1) was obtained for each of the diastereomeric systems **3**, **4** and

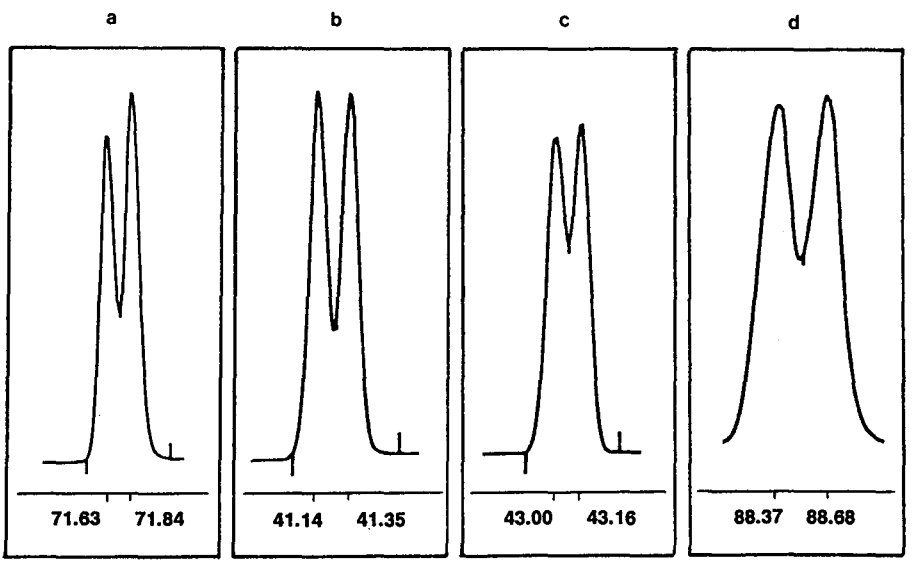



Fig. 1. Partial gas chromatograms illustrating resolution of the diastereomeric components of silyl acetals: (a) **3**, 60-m column; (b) **4**, 30-m column; (c) **5**, 30-m column; (d) **5**, 60-m column. Retention times of components are indicated in minutes.

**5**; doubling the column length increased the peak resolution in each instance (Table I). Peak splitting was not observed for the silyl acetals **1** and **2** [$R^2 = CH_3$, $R^3 = C_2H_5$, $n$-$C_3H_7$ (Table I)]. It seems that significant differences between the substituents ($R^2$ and $R^3$) are necessary if GLC resolution of the respective diastereomers is to be achieved and, although $^1H$ and $^{13}C$ NMR chemical shift non-equivalence was observed for all five of the systems examined, the signal doublings are more numerous and significant in the spectra of the silyl acetals **4** and **5** where the substituents are markedly different (*i.e.* $R^2 = CH_3$, $R^3 = C_6H_5$).

The results reflect a close correlation between GLC and $^1H$ NMR integral ratios and illustrate the potential of chiral silyl derivatization agents in the GLC analysis of enantiomers. Further research is expected to involve the development of more effective enantio-differentiating chiral silyl probes and extension to other functional groups.

ACKNOWLEDGEMENTS

The authors thank Mr. Ivan Antonowitz (CSIR) for obtaining the high-field NMR spectra, and Rhodes University and the Foundation for Research Development (CSIR) for generous financial support.

REFERENCES

1 P. T. Kaye and R. A. Learmonth, *Synth. Commun.*, 19 (1989) 2337.
2 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 167 (1978) 187.
3 W. A. König, P. Mischnick-Lubbecke, B. Brassat, S. Lutz and G. Wenz, *Carbohydr. Res.*, 183 (1988) 11.
4 W. A. König, W. Rahn and J. Eyem, *J. Chromatogr.*, 133 (1977) 141.
5 V. Schurig, in J. D. Morrison (Editor), *Asymmetric Synthesis, Vol. 1, Analytical Methods*, Academic Press, New York, 1983, p. 59.
6 C. Rosini, G. Uccello-Barretta, D. Pini, C. Abete and P. Salvadori, *J. Org. Chem.*, 53 (1988) 4579.
7 S. C. Benson, P. Cai, M. Colon, M. A. Haiza, M. Tokles and J. K. Snyder, *J. Org. Chem.*, 53 (1988) 5335.
8 P. Salvadori, G. Uccello-Barretta, S. Bertozzi, R. Settambolo and R. Lazaroni, *J. Org. Chem.*, 53 (1988) 5768.
9 P. Mangeney, F. Grojean, A. Alexakis and J. F. Normant, *Tetrahedron Lett.*, 29 (1988) 2675.
10 P. Mangeney, A. Alexakis and J. F. Normant, *Tetrahedron Lett.*, 29 (1988) 2677.
11 M. Tokles and J. K. Snyder, *Tetrahedron Lett.*,, 29 (1988) 6063.
12 M. E. Jung and K. T. Hogan, *Tetrahedron Lett.*, 29 (1988) 6199.
13 T. H. Chan and D. Wang, *Tetrahedron Lett.*, 30 (1989) 3041.
14 W. J. Richter, *J. Organomet. Chem.*, 169 (1979) 9.
15 T. H. Chan, Q.-J. Peng, D. Wang and J. A. Guo, *J. Chem. Soc., Chem. Commun.*, (1987) 325.

*Journal of Chromatography*, 503 (1990) 442–448
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 192

# Note

# Gas chromatographic analyses of hydroxyoxime extractants of metals and their intermediates

## I. Alkylsalicylaldehyde oximes

JACEK STANIEWSKI and JAN SZYMANOWSKI*

*Poznań Technical University, Institute of Chemical Technology and Engineering, Pl. M. Skłodowskiej-Curie 2, 60-965 Poznań (Poland)*

(First received September 22nd, 1989; revised manuscript received December 6th, 1989)

Alkyl derivatives of salicylaldehyde oximes, produced by Acorga (Acorga P-50) and Henkel (LIX 860), are some of the most important copper extractants, used in some old and new plants[1–4]. According to the manufacturers, Acorga P 50 contains 2-hydroxy-5-nonylbenzaldehyde oxime and LIX 860 contains 2-hydroxy-5-dodecylbenzaldehyde oxime. The Acorga reagents Acorga P-5100, P-5200 and P-5300 are blends of P-50 and nonylphenol with weight ratios of 1:1, 1:2 and 1:3, respectively, and Acorga PT-5050 is a 2:1 blend of P-50 and tridecanol. Apart from LIX 860, Henkel reagents containing 2-hydroxy-5-dodecylbenzaldehyde oxime are LIX 622, a mixture of LIX 860 and tridecanol, LIX 984, a 1:1 blend of LIX 860 and LIX 84 (2-hydroxy-5-nonylacetophenone oxime), and LIX 865, a mixture of LIX 860 and LIX 65N (2-hydroxy-5-nonylbenzophenone oxime).

These extraction reagents are diluted to approximately 50% with aliphatic–aromatic solvents. The disadvantage of the alkyl derivatives of salicylaldehyde oximes is a faster hydrolysis to aldehydes in strongly acidic conditions in relation to standard LIX 65N. As a result, appropriate aldehydes can be present in commercial products and they can be formed during extraction[5].

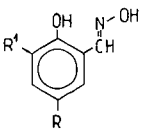
2-Hydroxy-5-alkylbenzaldehyde oximes have not been analysed by gas chromatography (GC). High-performance liquid chromatography (HPLC) has been used for the determination of nonylphenol, aldehydes and oximes in the commercial Acorga series P-5000[6]. GC and/or HPLC have been used for the identification and determination of other aliphatic α-hydroxyoximes (LIX 63) and some aromatic β-hydroxyoximes (all of the above 2-hydroxybenzophenone derivatives)[7–10].

The aim of this work was to obtain some analytical data for model and commercial hydroxyoxime metal extractants (alkyl derivatives of salicylaldehyde oxime) on some different phases, to investigate the relationships between the arithmetic retention indices of these compounds and their structures and to calculate increments of the retention indices for some characteristic groups present in these compounds.

0021-9673/90/$03.50 

## EXPERIMENTAL

Individual model derivatives of salicylaldehyde oxime[11] of the following structure:



where R denotes H, methyl ethyl, *n*-butyl, *n*-hexyl, *n*-octyl, *n*-dodecyl, *tert.*-butyl and 1,1,3,3-tetramethylbutyl, (*tert.*-octyl) and $R^1$ denotes H, Cl, *tert.*-butyl and $NO_2$ were analysed in the form of trimethylsilyl derivatives. Two commercial extractants, Acorga P 5100 and P 5300, were also analysed.

A sample of 10–20 mg was treated with 0.2 $cm^3$ of N,O-bis(trimethylsilyl) acetamide (BSA) or N,O-bis(trimethylsilyltrifluoroacetamide (BSTFA) at 50°C for 30 min.

Gas chromatographic analyses were carried out with a JEOL Model JGC 1100 TFP instrument equipped with a flame ionization detector. The glass columns were filled with 3% SE-30 on Gas-Chrom Q (100–120 mesh) (column I), 3% OV-210 on Gas-Chrom Q (120–140 mesh) (column II) or 3% OV-225 on Gas-Chrom Q (120–140 mesh) (column III). Column I was 2 m long and columns II and III 1.8 m long, each of I.D. 3 mm. Helium was used as the carrier gas at a flow-rate of 40 $cm^3$/min. The injector and detector temperatures were 290°C. The oven temperature was increased linearly at 4°C/min from 80 to 280°C for column I and from 50 to 250°C for columns II and III.

Silylated samples of 0.1-$\mu$l volume were injected and the arithmetic retention indices were calculated using $C_{10}$–$C_{24}$, $C_{26}$, $C_{28}$ and $C_{32}$ *n*-alkanes as reference standards. In those instances when a compound in the sample was not separated or was separated incompletely from an *n*-alkane, this standard was omitted from the mixture and its retention time was calculated from its relative retention time in another mixture.

Commercial extractants were analysed using column I. The weight ratio of nonylphenol to 2-hydroxy-5-nonylbenzaldehyde oxime was determined from peak surface areas assuming correction coefficients to be equal to 1. The peaks of 2-hydroxy-5-alkylbenzaldehyde oxime and nonylphenol in commercial Acorga extractants were identified by comparison with chromatograms of nonylphenol and the hydroxyoxime obtained from intermediate nonylphenol.

## RESULTS AND DISCUSSION

Derivatives of salicylaldehyde oximes have two active groups *i.e.*, a hydroxyl phenolic group and an oximino group. Each of these two groups can react with a silylation agent to form appropriate trimethylsilyl derivatives:

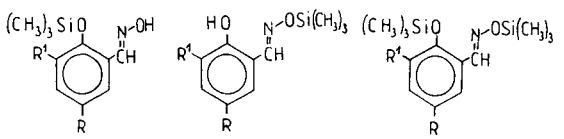


TABLE I

INFLUENCE OF DIFFERENT TEMPERATURE PROGRAMMES ON ARITHMETIC RETENTION INDICES ON SE-30

Maximum standard deviation = 0.3

| *Compound* | *Temperature programme [starting temperature (°C)/ rate of increase (°C/min)]* | | | |
|---|---|---|---|---|
| | *50/6* | *60/4* | *80/4* | *100/3* |
| 2,4-Di-*tert.*-butylphenol | 1539.6 | 1538.3 | 1538.7 | 1537.6 |
| 4-*tert.*-Octylphenol | 1630.9 | 1628.7 | 1629.2 | 1628.1 |
| 2-Hydroxy-5-*tert.*-butylbenzaldehyde oxime | 1762.4 | 1763.3 | 1763.1 | 1763.3 |
| 2-Hydroxy-5-*tert.*-butylbenzaldehyde | 1881.9 | 1877.6 | 1878.6 | 1875.5 |
| 2-Hydroxy-3,5-di.*tert.*-butylbenzaldehyde oxime | 1936.6 | 1936.2 | 1936.7 | 1936.1 |
| 2-Hydroxy-3-chloro-5-*tert.*-octylbenzaldehyde oxime | 2106.9 | 2104.5 | 2104.7 | 2102.9 |

As a result, three peaks may be observed on chromatograms. However, under the experimental conditions used for silylation (after a reaction time of 30 min), only one peak of the appropriate di(trimethylsilyl) derivative was observed and its size and shape did not change after further silylation. The structure of the di(trimethylsilyl) derivative was confirmed by gas chromatography–mass spectrometry.

The effect of various temperature programmes on the arithmetic retention index for the compounds considered is small and can be neglected (Table I). The maximum standard deviation is 0.3.

The determined values of the arithmetic retention index are given in Table II, and the relationships between retention indices determined on OV-210 and OV-225

TABLE II

VALUES OF ARITHMETIC RETENTION INDICES

| *Group* | | *Stationary phase* | | |
|---|---|---|---|---|
| *R* | $R^1$ | *SE-30* | *OV-210* | *OV-225* |
| H | H | 1560 | 1739 | 1803 |
| Methyl | H | 1627 | 1793 | 1864 |
| Ethyl | H | 1693 | 1849 | 1925 |
| *n*-Butyl | H | 1852 | 2004 | 2081 |
| *n*-Hexyl | H | 2031 | 2185 | 2258 |
| *n*-Octyl | H | 2216 | 2369 | 2445 |
| *n*-Dodecyl | H | 2606 | 2745 | 2827 |
| *tert.*-Butyl | H | 1763 | 1909 | 1966 |
| *tert.*-Butyl | *tert.*-Butyl | 1937 | 2013 | 2051 |
| *tert.*-Butyl | Cl | 1873 | 2026 | 2066 |
| *tert.*-Octyl | H | 1995 | 2144 | 2171 |
| *tert.*-Octyl | Cl | 2105 | 2267 | 2271 |
| *tert.*-Octyl | $NO_2$ | 2241 | 2671 | 2606 |

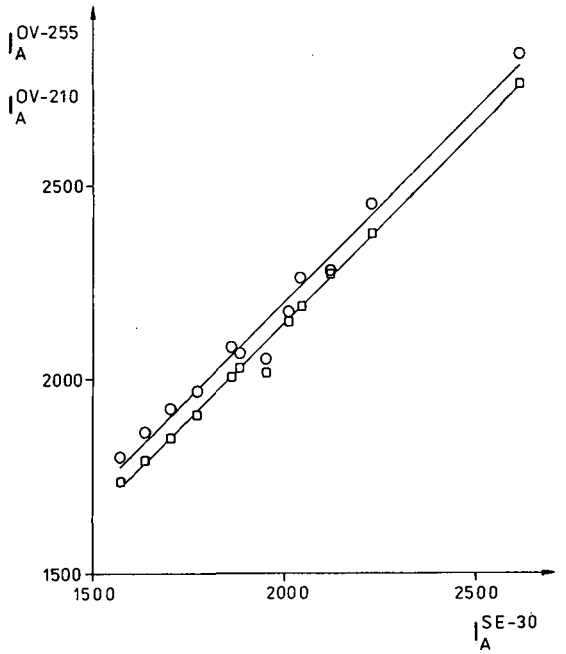



Fig. 1. Relationship between arithmetic retention indices on (□) OV-210 or (○) OV-225 and arithmetic retention indices on SE-30.

and those on SE-30 are presented in Fig. 1. The phases used for the analysis of both the commercial and model hydroxyoxime extractants were considered.

The retention indices of 2-hydroxy-5-alkylbenzaldehyde oximes on OV-210 and OV-225 are higher than those on the non-polar SE-30 by *ca.* 155 and 222 units, respectively. The following statistically significant linear relationships were obtained:

$$I_A^{OV-210} = 0.977 I_A^{SE-30} + 194 \qquad R^2 = 0.9926$$

$$I_A^{OV-225} = 0.978 I_A^{SE-30} + 249 \qquad R^2 = 0.9824$$

where $R^2$ is the determination coefficient (the square of the correlation coefficient).

Statistically significant linear relationships are also observed between the arithmetic retention indices of 2-hydroxy-5-*n*-alkylbenzaldehyde oximes and the number of carbon atoms ($n$) in the alkyl group. They are as follows on SE-30, OV-210 and OV-225, respectively:

$$I_A^{SE-30} = 94.5n + 1468 \qquad R^2 = 0.9996$$

$$I_A^{OV-210} = 92.7n + 1631 \qquad R^2 = 0.9999$$

and

$$I^{OV-225} = 93.5n + 1702 \qquad R^2 = 0.9999$$

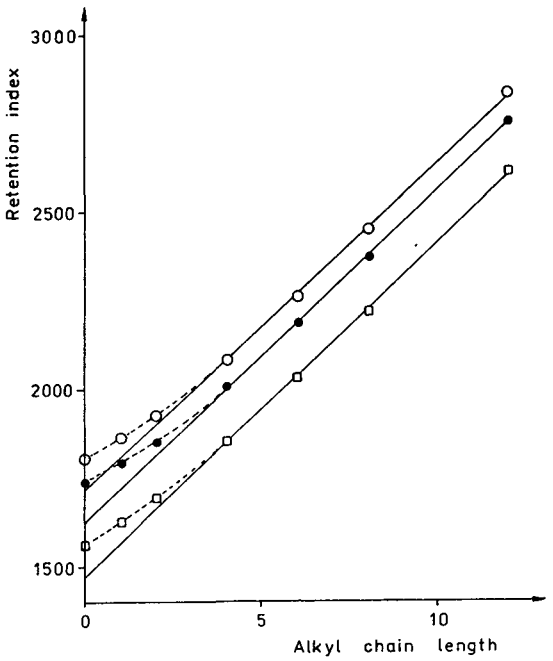



Fig. 2. Relationship between arithmetic retention indices of 2-hydroxy-5-*n*-alkylbenzaldehyde oximes on (□) SE-30, (●) OV-210 and (○) OV-225 and length of alkyl chain.

Hence, the determined increments of the methylene/methyl group (95, 93 and 94) are approximately the same and near to the theoretical value of 100.

Some important deviations from these linear relationships are observed for homologues having a short alkyl chain, *i.e.*, containing less than four carbon atoms (Fig. 2). These deviations increase as the length of the alkyl chain decreases, and they disappear for oximes with a butyl group.

The data in Table II also demonstrate that the chlorine atom at the 3-position, *i.e.*, in the direct neighbourhood of the phenolic group, increases the retention index by approximately 110, 120 and 100 units on SE-30, OV-210 and OV-225, respectively, and the effect of the alkyl chain length (*tert.*-butyl or *tert.*-octyl) is relatively small.

The presence of a *tert.*-butyl group at the 3-position increases the retention index by 174, 104 and 85 units on these three phases, respectively, while the replacement of an *n*-butyl group with a *tert.*-butyl group decreases the retention index by 84, 95 and 115 units, respectively. A much stronger effect is observed when the *n*-octyl group is replaced with a *tert.*-octyl group, which contains mainly 1,1,3,3-tetramethylbutyl; the retention index decreases by 221, 225 and 274 units on SE-30, OV-210 en OV-225, respectively.

A nitro group at the 3-position strongly increases the retention index by 246, 527 and 435 units, respectively.

A typical chromatogram of the commercial extractant Acorga P 5100 is given in Fig. 3. Two groups of components were separated which were identified as nonylphenol (arithmetic retention indices above 1700 with a range of about 70 units) and

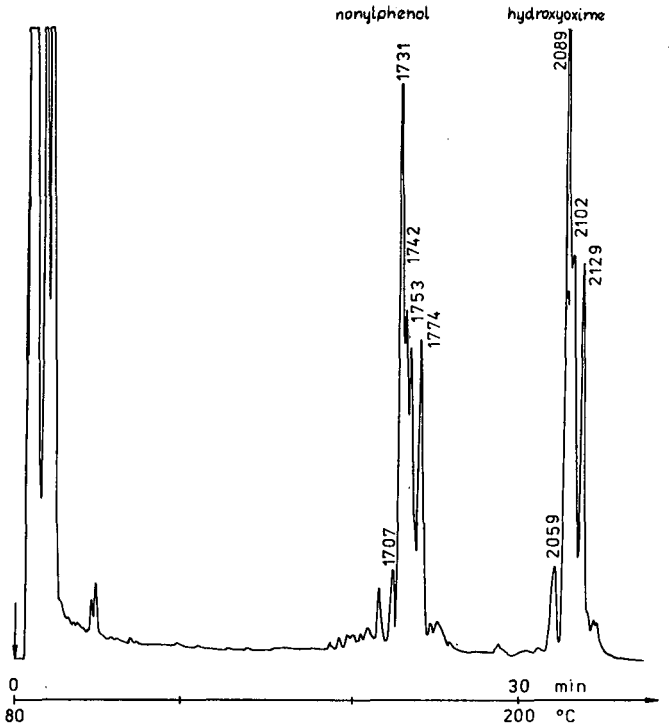



Fig. 3. Chromatogram of commercial extractant Acorga P 5100.

2-hydroxy-5-nonylbenzaldehyde oxime (arithmetic retention index of about 2100 with a range of 70 units).

The separation of several components is caused by the complex composition of the commercial nonylphenol obtained by phenol alkylation with propylene trimer. As a result, the nonyl group is highly branched and nonylphenol contains several iso-

TABLE III

WEIGHT RATIO OF NONYLPHENOL TO 2-HYDROXY-5-NONYLBENZALDEHYDE OXIME IN COMMERCIAL EXTRACTANTS P 5100 AND P 5300

| *Analysis No.* | *P 5100* | *P5300* |
|---|---|---|
| 1 | 0.88 | 3.10 |
| 2 | 0.98 | 3.31 |
| 3 | 0.87 | 2.82 |
| 4 | 0.98 | 2.92 |
| 5 | 0.89 | 2.82 |
| 6 | 0.94 | 3.30 |
| 7 | 0.93 | 2.93 |
| Average | 0.93 | 3.03 |

mers. Moreover, it also contains other groups of components, including 2-alkylphenol and small amounts of O-alkylation products[12].

The determined weight ratios of nonylphenol to 2-hydroxy-5-nonylbenzaldehyde in Acorga P 5100 and P 5300 (Table III) are in good agreement with the values given by manufacturer, *i.e.*, 1:1 and 3:1 in P 5100 and P5300, respectively.

## CONCLUSIONS

Linear relationships are observed between retention indices on OV-210 and OV-225 and on SE-30 for 2-hydroxy-5-alkylbenzaldehyde oximes and their derivatives substituted at the 3-position. The retention indices of 2-hydroxy-5-*n*-alkylbenzaldehyde oximes increase linearly as the length of the alkyl group increases. Some deviations from linear relationships are observed only for the first homologues containing less than four carbon atoms in the alkyl group.

The increments of various structural groups present in the compounds considered can be used to estimate their retention indices on phases considered.

Chromatographic analyses confirm the weight ratio of nonylphenol to 2-hydroxy-5-nonylbenzaldehyde oxime in commercial extractants P 5100 and P 5300 as 1:1 and 3:1, respectively.

## ACKNOWLEDGEMENT

This work was supported by Polish Research Program CPBP 03.08.

## REFERENCES

1 P. R. Danesi and R. Chiarizia, *CRC Crit. Rev. Anal. Chem.*, 10 (1980) 1–126.
2 J. F. C. Fisher and G. W. Notebart, in T. C. Lo, M. H. I. Baird and C. Hanson (Editors), *Handbook of Solvent Extraction*, Wiley, New York, 1983, pp. 649–665.
3 J. Szymanowski, *Wiad. Chem.*, 38 (1984) 371–399.
4 J. Szymanowski, in *Proceedings of the Annual Scientific Meeting of the Polish Chemical Society, Poznań, Poland*, Poznań University, Poznań, 1985, pp. 48–70.
5 R. J. Whewell, H. J. Foakes and M. A. Hughes, *Hydrometallurgy*, 7 (1981) 7–26.
6 I. C. Stone, T. N. Anderson, J. C. Parr and D. D. Metcalf, *Anal. Chem.*, 54 (1982) 1965–1968.
7 A. W. Ashbrook, *J. Chromatogr.*, 105 (1975) 151–156.
8 W. Sowa, B. H. Licht and I. J. Itzkovitch, *J. Chromatogr.*, 116 (1976) 197–200.
9 J. W. Hosking and N. M. Rice, *Hydrometallurgy*, 3 (1978) 217–231.
10 E. Y. O. Paatero, *Hydrometallurgy*, 13 (1984) 193–201.
11 D. Stepniak-Biniakiewicz and J. Szymanowski, *Hydrometallurgy*, 7 (1981) 299–313.
12 V. Gerhardt and H. Much, *Tenside Deterg.*, 18 (1981) 120–124.

*Journal of Chromatography*, 503 (1990) 449–452
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 223

# Note

## Use of non-ionic and zwitterionic surfactants to enhance selectivity in high-performance capillary electrophoresis

## An apparent micellar electrokinetic capillary chromatography mechanism

SALLY A. SWEDBERG
*Hewlett-Packard Laboratories, Building 28C, P.O. Box 10490, Palo Alto, CA 94303-0969 (U.S.A.)*
(First received September 1st, 1989; revised manuscript received December 15th, 1989)

High-performance capillary electrophoresis (HPCE) has initially attracted much attention as a high-efficiency separation technique. Within separations science, the impact of efficiency and selectivity in achieving resolution is often discussed. As a newly emerging technique, one question surrouding open tubular HPCE is whether or not it is an inherently high-efficiency, low-selectivity process.

Terabe *et al.*[1] have demonstrated that selectivity can be added to open tubular HPCE by using ionic surfactants in the micellar electrokinetic capillary chromatography (MECC) format. Here, at or above the critical micelle concentration (CMC) of the surfactant, micelles act as a pseudophase. Based on the difference in partitioning of various solute molecules between the pseudophase and bulk solvent and the difference in mobility of the electroosmotic flow (EOF) of the bulk solvent *vs.* the mobility of the ionic pseudophase, separation is achieved.

What we have observed in our laboratory is the enhancement of separation of closely related species by the use of non-ionic and zwitterionic surfactants. These surfactants are only effective in achieving separations at or above the CMC. Examples are given and an explanation for the observation is proposed.

## EXPERIMENTAL

The apparatus used has previously been described[2]. The capillaries used were aryl pentafluoro (APF) deactivated capillaries[3] with inner diameters ranging from 20 to 50 $\mu$m.

The octyl glucoside and CHAPS ({3-[3-(chloroamidopropyl) dimethylammonio]-1-propanesulfonate}) used were obtained from Calbiochem (San Diego, CA, U.S.A.). For the octyl glucoside, both the Ultrol and regular grade were evaluated. In this particular application, the regular grade was found to be suitable. Desipramine and nortriptyline were purchased from Sigma (St. Louis, MO, U.S.A.). The peptides were obtained from Peninsula Labs. (Belmont, CA, U.S.A.).

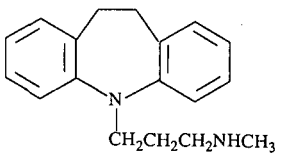
Specific conditions for each experiment are noted in the figure legends.

0021-9673/90/$03.50 

RESULTS AND DISCUSSION

Two specific examples of selectivity enhancement by using non- or zwitterionic surfactants will be used:

(1) The separation of the tricyclic antidepressant desipramine from a closely related substance, nortriptyline.




Desipramine


Nortriptyline

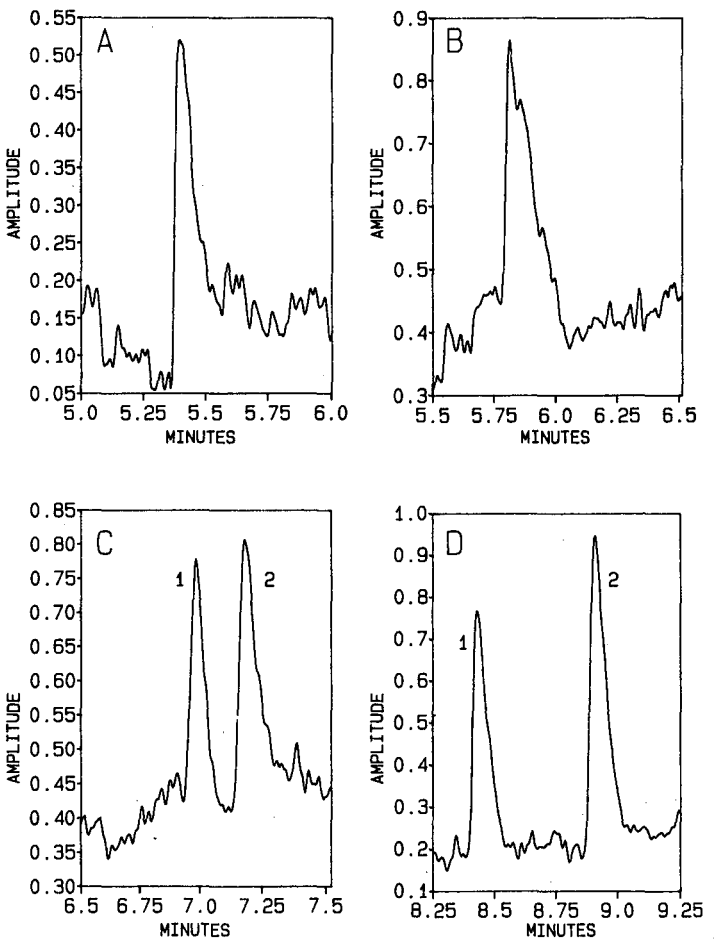



Fig. 1. Separation of desipramine and nortriptyline as a function of increasing amounts of octyl β-D-glucoside in 67 m*M* phosphate (100 m*M* $Na^+$) pH 7.0 buffer. (A) No octyl glucoside; (B) 10 m*M* octyl glucoside; (C) 20 m*M* octyl glucoside (CMC); (D) 30 m*M* octyl glucoside (above AN). Conditions: 50 μm I.D. × 375 μm O.D. fused-silica APF capillary, 60 cm to detection; detection: 213 nm on-column; field strength: 300 V/cm. Peaks: 1 = desipramine; 2 = nortriptyline.

(2) The separation of heptapeptides angiotensin III and [Val$^4$]-angiotensin III. These two peptides differ in the substitution of isoleucine for valine at the fourth residue from the N-terminus.

Micelle formation is affected by numerous variables including pH, ionic strength and temperature. Initial studies were done under defined conditions of sodium ion concentration and pH where CMC and aggregation number (AN) are known for the surfactants used[4].

In Fig. 1, at 100 m$M$ sodium ion and pH 7.0, the electropherogram panel shown, establishes that the separation of desipramine from nortriptyline occurs at CMC and improves at the AN for octyl glucoside. The experiment was repeated with CHAPS with similar results.

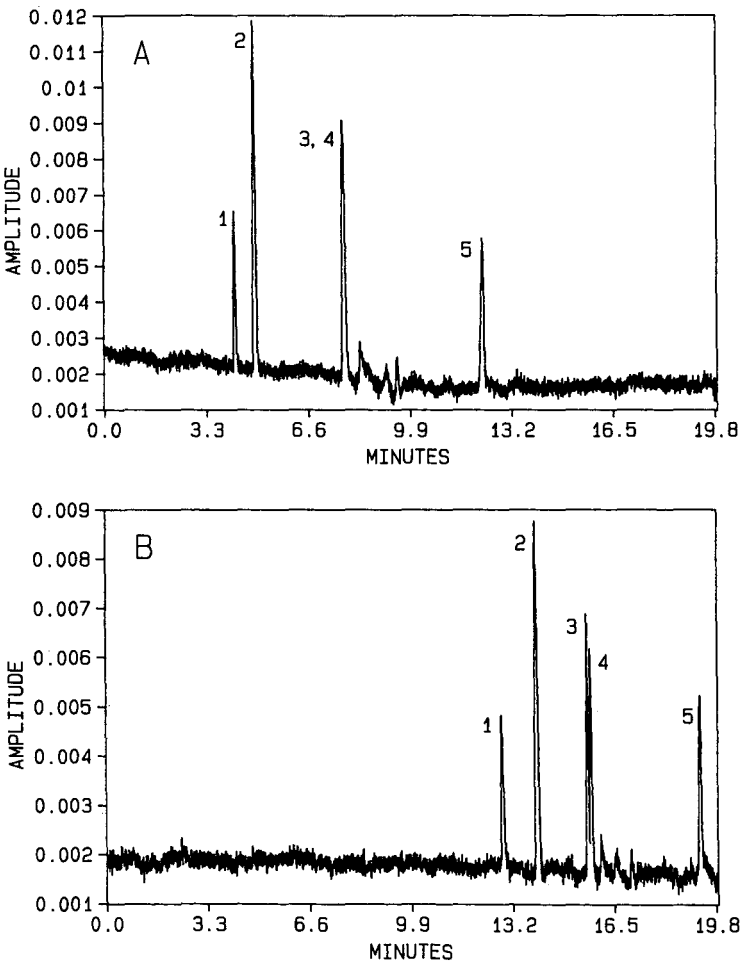



Fig. 2. Enhancement of selectivity is demonstrated in (B) by the addition of 80 m$M$ octyl glucoside to 250 m$M$ phosphate (pH 7.0) electrophoresis buffer. Peaks: 1 = bradykinin; 2 = releasing luteinizing hormone; 3 = [Val$^4$]-angiotensin III; 4 = angiotensin III; 5 = angiotensin II. Conditions: 17 $\mu$m I.D. × 375 $\mu$m O.D. fused-silica APF capillary, 70 cm to detection; detection: 210 nm on-column; field strength: 250 V/cm.

Fig. 2 shows electropherograms comparing separations of six peptides before and after addition of octyl glucoside. The addition of 80 m*M* octyl glucoside to the electrophoresis buffer notably affected selectivity; particularly of the angiotensin III pair.

Separation by MECC, as was previously mentioned, depends on both the partitioning of solute between the bulk solvent and the micelle pseudophase, and also the difference in electrophoretic mobility of the bulk solvent *vs.* the micelle. For non-ionic or zwitterionic surfactants, even though these surfactants bear no net charge, the explanation for the mobility of micelles formed from such surfactants fits perfectly within what is known about double ion layer formation on unionized surfaces. It is known from colloid and surface science that the three major charging mechanisms for a surface in contact with a polar medium are ionization, ion adsorption and ion dissolution[5]. For the suspended non-ionic or neutral ionic micellar structures, ion adsorption giving rise to a mobility in an applied field is as predictable as the formation of an electric double layer in Teflon capillary giving rise to an electroosmotic flow in that material.

The potential advantages of non- or zwitter-ionic surfactants for use in HPCE include:

(1) These materials have much less impact on the magnitude of the EOF mobility than the charged surfactants were observed to have had. Unlike CTAB, they cannot reverse the EOF direction.

(2) These materials are known to have little impact on protein structure as witnessed by excellent recovery of biological activity when those surfactants are used[4].

Uses of these surfactants for macromolecular separations are currently being explored by this laboratory.

REFERENCES

1 S. Terabe, S. K. Otsuka, K. Ichikawa, K. A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1985) 111–113.
2 H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
3 S. A. Swedberg, *Anal. Biochem.*, in press.
4 J. Neugebauer, *A Guide to the Properties and Use of Detergents in Biology and Biochemistry, Document No. 8183-687*, Calbiochem Corporation, San Diego, CA.
5 D. J. Shaw, *Introduction to Colloid and Surface Chemistry*, Butterworths, London, 1975, pp. 133–135

*Journal of Chromatography*, 503 (1990) 453–458
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 193

# Note

## Determination by high-performance liquid chromatography of the binding properties of charged β-cyclodextrin derivatives with drugs

NICOLE THUAUD, BERNARD SEBILLE*, ANDRÉ DERATANI and GÉRARD LELIEVRE
*Laboratoire de Physico-Chimie des Biopolymères, Unité Mixte No. 27, CNRS, Université Paris Val de Marne, 2–8 Rue Henri Dunant, 94320 Thiais (France)*
(First received August 3rd, 1989; revised manuscript received December 5th, 1989)

One of the most important properties of cyclodextrins is their ability to form inclusion complexes with numerous molecules that they take up in their central hydrophobic cavity. The selective chemical modification of cyclodextrins (CDs) provides opportunities to modify their complexing behaviour. For instance, the introduction of a hydrophobic surface to the parent CD may enhance considerably their association constants with several guests[1]. Tabushi *et al.*[2] used this to prepare a 'molecular design for artificial enzymes'. Moreover, the specific binding of anionic guests, such as drugs to aminated cyclodextrins in which both hydrophobic and electrostatic interactions participate, has been reported[3–9]. The binding and catalytic properties of these derivatives are stronger than those of the parent CD.

CDs are used in pharmaceutical research essentially for improving drug stability, dissolution rates and bioavaibility[10]. However, the application of β-CDs in the pharmaceutical field depends on their aqueous solubility. Thus various functional groups have been incorporated in the CD molecules. Methylated[11–15] and hydroxyalkylated[15] derivatives have been prepared and improved the solubilizing and binding properties for drugs compared with the parent CD.

This work was undertaken in order to study the complexing behaviour towards basic, acidic and neutral drugs of two β-CD derivatives, highly soluble in water and bearing either negative or positive electric charges.

## EXPERIMENTAL

### *Materials*

β-CD hydrate was a gift from Roquette (Lestrem, France). The drugs were purchased from Sigma (St. Louis, MO, U.S.A.)

### *Preparation of the sodium salt of the carboxymethyl ether of β-CyD (β-CyDCME)*

This derivative was prepared according to the method of Lammers *et al.*[17] in alkaline solution with monochloroacetic acid. From an initial 15 g of β-CD hydrate, 9

0021-9673/90/$03.50 

g of derivative were recovered after twice precipitating with methanol and drying at 90°C under vacuum. The product was free from sodium chloride (C < 0.1% by elemental analysis); 3.16 ± 0.20 anionic substituents per molecule of derivative were measured by titration of carboxylate groups with perchloric acid in acetic acid.

*Preparation of the hydroxypropyltrimethylammonium ether of β-CyD (β-CyDN$^+$)*

This cationic derivative was prepared in our laboratory according to the patented method of Parmerter *et al.*[18] and modified by Deratani *et al.*[19]. The results of elemental analysis (C 45.7, H 7.58, N 3.69, C 8.99, Na < 0.1%) confirmed the results of argentimetry, which gave 4.6 monosubstituents per molecule, and absence of salt. The molecular weight is thus 1832.

*HPLC apparatus*

The apparatus was the same as that used previously[16]. The columns were packed with commercial LiChrosorb Diol (10-μm particle diameter and 100 A pore diameter) support (Merck, Darmstadt, F.R.G.).

Several 10-cm long columns were necessary to separate the drug from the β-CD derivative, depending on the drug and on its concentration in the mobile phase; therefore three columns were used for some hydrocortisone experiments, whereas one or two were sufficient in the other instances.

A Waters Assoc. Model 401 difference refractometer was used for detecting β-CD and its derivatives. A Beckman Model 103 variable-wavelength UV detector was used for drug monitoring.

*Binding measurements*

These were done using the Hummel and Dreyer[20] method adapted by us for β-CD–drug binding studies with high-performance liquid chromatographic (HPLC) columns[16].

The columns were equilibrated and eluted with successive concentrations of the drug under study in 0.1 *M* phosphate buffer (pH 7.4). These concentrations were varied in the range $1 \cdot 10^{-5}$–$7 \cdot 10^{-4}$ *M* for hydrocortisone and $6 \cdot 10^{-5}$–$3 \cdot 10^{-3}$ *M* for the other drug.

Small aliquots of β-CD or its derivatives were injected into the column, usually 50 μl of 2 g/l solutions. However, in the hydrocortisone binding measurements, 0.2 g/l CD solutions were used and gave narrow, well separated peaks, because of the higher binding capacity of the host molecules for this drug.

## RESULTS AND DISCUSSION

Both β-CD derivatives are eluted at about the void volume of the columns, like β-CD itself, as monitored by refractive index (RI) detection (Fig. 1). It is easy to compare the binding capacity of the various host molecules to one drug from observation of the negative peak in Fig. 2a or b: a known molar amount of injected β-CDCME binds fewer molecules of indomethacin (or warfarin) than does β-CD, whereas β-CDN$^+$ binds more (Fig. 2a). Opposite effects are observed with propranolol (Fig. 2b).

The negative peak areas in Fig. 2a and b allow one to calculate according to a

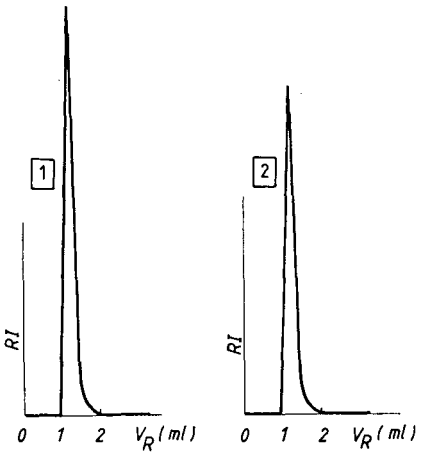



Fig. 1. Chromatograms of β-CD and β-CD derivatives on a 10 cm x 4.7 cm I.D. column packed with LiChrosorb Diol. Eluent, 0.1 M phosphate buffer (pH 7.4). Samples: 50 μl of 8 g/l solutions in phosphate buffer; differential refractometer, sensivity 32 ×. Peaks : 1 = β-CD; 2 = β-CDCME or β-CyDN$^+$. $V_R$ = Retention volume.

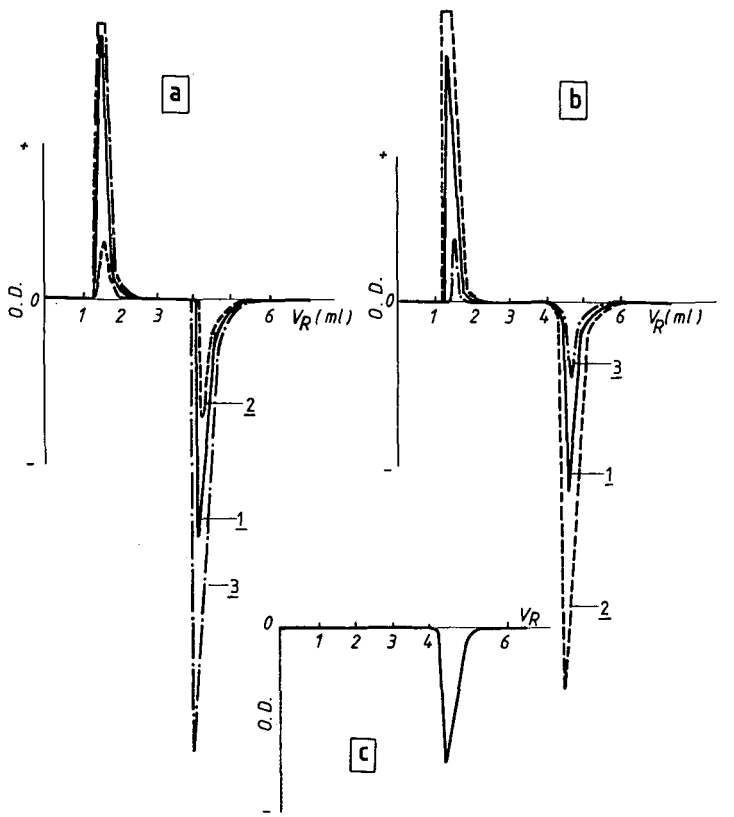



Fig. 2. Hummel and Dreyer[20] chromatograms obtained for inclusion complexation measurements of (a) warfarin and (b) propranolol on a 10-cm long column. Eluent, $10^{-4}$ *M* solution of the drug in phosphate buffer; all samples injected as 50 μl of 3.3 m*M* solutions. Peaks: 1 = β-CD; 2 = β-CyDCME; 3 = β-CyDN$^+$. (c) Calibration obtained by injection of 50 μl of phosphate buffer under the above experimental conditions; the obtained peak gives the signal of a deficit in $5 \cdot 10^{-9}$ mol of drug.

prior calibration obtained by injecting pure buffer (Fig. 2c), the amount of drug that has been complexed by the host molecule. The area of the positive peak, where the host molecule and its inclusion complex emerge, is not identical with that of the negative peak, because of the difference in UV molar absorption for the bound and unbound drug.

It must be emphasized that the HPLC technique used here requires only small amounts of β-CD derivatives for the determination of their complexing properties. This is advantageous over other methods based on solubility or spectroscopic experiments. Some β-CD binding constants measured previously by these methods have been reported for comparison and validation of the HPLC method[16]. However, there are no results in the literature concerning the binding of β-CD derivatives.

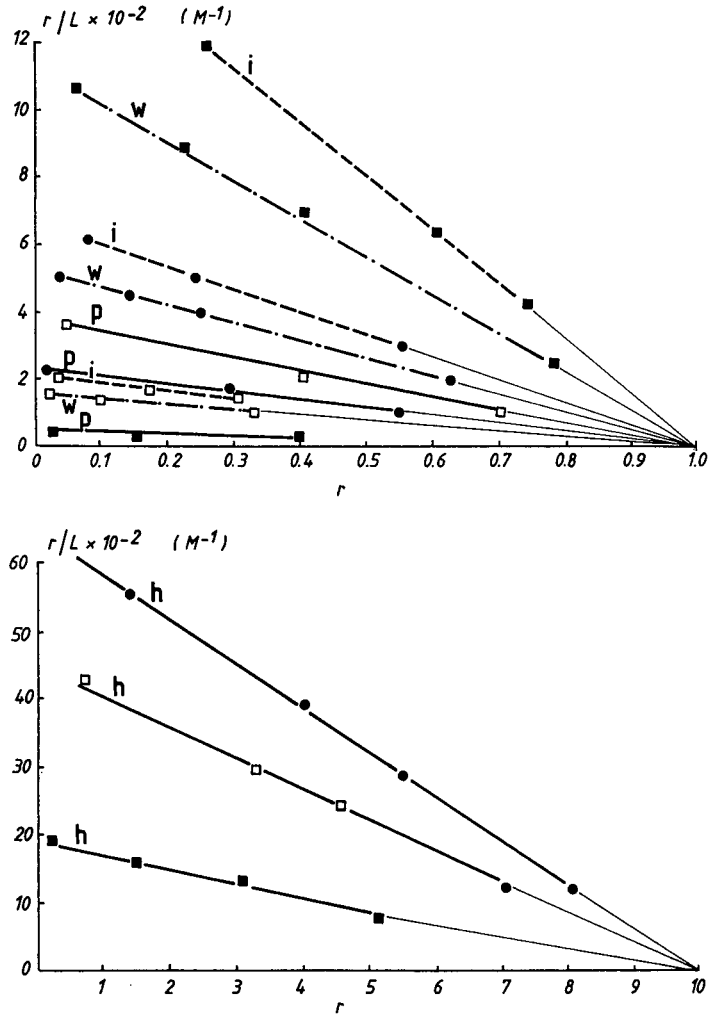



Fig. 3. Scatchard plots obtained for complexation of β-CD derivatives with drugs: h = hydrocortisone; i = indomethacin; w = warfarin; p = propranolol. Host molecules: ●, β-CD; ■, β-CyDN$^+$; □, β-CDCME. $r$ = Guest/host molar binding ratio.

TABLE I

ASSOCIATION CONSTANTS OF DRUG–β-CD DERIVATIVE COMPLEXES AT pH 7.4
The ± values are confidence intervals.

| *β-CD* | *Warfarin* ($pK_a$ = 5.1) | *Indomethacin* ($pK_a$ = 4.5) | *Propranolol* ($pK_a$ = 9.45) | *Hydrocortisone* |
|---|---|---|---|---|
| β-CD | 520 ± 30 | 620 ± 50 | 220 ± 20 | 6200 ± 50 |
| β-CDCME | 150 ± 10 | 250 ± 20 | 400 ± 40 | 4600 ± 50 |
| β-CDN$^+$ | 1150 ± 50 | 1500 ± 50 | 50 ± 10 | 2000 ± 50 |

Warfarin and indomethacin are acidic drugs, with p$K_a$ 5.1 and 4.5, respectively. They are negatively charged under the experimental pH conditions, whereas propranolol, a basic drug with p$K_a$ 9.5, is positively charged under these conditions. Hence β-CDN$^+$ has a greater binding capacity than β-CD for negatively charged drugs and a smaller capacity for positively charged drugs. Opposite effects are observed for β-CyDCME. There is evidence for attractive electrostatic interactions between oppositively charged hosts and guests and repulsive effects between identically charged couples.

The Scatchard plots[21], obtained by modifying the concentration of the eluting drug solution (L) are presented in Fig. 3. They converge towards the abscissa at a value of 1, indicating the formation of only 1:1 inclusion complexes, as is usually the case for inclusion of guests in β-CD. The association constants were determined from the slopes of the straight lines, and are reported in Table I. A 2–3-fold increase in their values is observed in the enhanced binding of β-CD derivatives with respect to β-CyD itself. The order of magnitude of the observed phenomena is the same as that reported by Matsui *et al.*[9] between charged dyes and sulphonato and pyridino β-CD derivatives.

The present results provide additional new examples of inclusion complex stabilization by the formation of cooperative electrostatic interactions. Other experiments have been carried out concerning the complexation of hydrocortisone, a non-charged hydrophobic drug.

A decrease in the stability of the inclusion compound formed with β-CD is observed with the quaternary ammonium ether derivative (Table I). A decreased hydrophobicity of the β-CD molecule, due to the presence of the substituent groups, is probably responsible for this phenomenon. This result has to be compared with the decrease in stability of the inclusion complex formed between the undissociated form of an azo dye and a trimethylammonium β-CD derivative, reported previously[4]. Such correlations between the stabilities of the complexes and the structure of the host CD derivatives permit approaches to the design of drug-complexing molecules.

REFERENCES

1 I. Tabushi, K. Fujita and H. Kawakubo, *J. Am. Chem. Soc.*, 99 (1977) 6456.
2 I. Tabushi, Y. Kuroda and H. Shimokawa, *J. Am. Chem. Soc.*, 101 (1979) 1614.
3 B. Siegel and R. Breslow, *J. Am. Chem. Soc.*, 97 (1975) 6969.
4 Y. Matsui and A. Okimoto, *Bull. Chem. Soc. Jpn.*, 51 (1978) 3030.
5 J. Boger, P. G. Brenner and J. R. Knowles, *J. Am. Chem. Soc.*, 101 (1979) 7631.

6 I. Tabushi, *J. Am. Chem. Soc.*, 101 (1979) 1614.
7 Z. Goren, P. Dan and I. Willner, *Chem. Lett.*, (1984) 845.
8 A. Ueno, F. Moriwaki, T. Osa, F. Hamada and K. Murai, *Tetrahedron Lett.*, 26 (1985) 899.
9 Y. Matsui, K. Ogawa, S. Mikawi, M. Yoshimoto and K. Mochida, *Bull. Chem. Soc. Jpn.*, 60 (1987) 1219.
10 D. Duchene in D. Duchene (editor), *Cyclodextrins and Their Industrial Uses*, Edition de Santé, Paris, 1987, p. 2.
11 K. Uekama and T. Irie, *Pharm. Int.*, 6 (1985) 61.
12 B. W. Muller and U. Brauns, *Int. J. Pharm.* 26 (1985) 77.
13 J. Pitha, *Life Sci.*, 29 (1981) 307.
14 Y. Nakai, K. Yamamoto, K. Terada and N. Moribe, *Chem. Pharm. Bull.*, (1982) 1796.
15 J. Szejtli, *J. Inclus. Phenom.*, 1 (1983) 135.
16 B. Sebille, N. Thuaud, J. Piquion and N. Behar, *J. Chromatogr.*, 409 (1987) 61.
17 J. N. J. J. Lammers, J. L. Koole and J. Hurkmans, *Starke*, 23 (1971) 167.
18 S. M. Parmerter, E. E. Allen and G. R. Hull, *U.S. Pat.*, 3 453 257, 1969.
19 A. Deratani, G. Lelievre, T. Maraldo and B. Sebille, *Carbohydr. Res.*, 192 (1989) 215.
20 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 63 (1962) 530.
21 G. F. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.

*Journal of Chromatography*, 503 (1990) 459–465
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 171

# Note

## Semi-automated method for the determination of abscisic acid in crude plant extracts

ANNA M. CARRASQUER, ISIDRE CASALS*[a] and LEONOR ALEGRE
*Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028-Barcelona (Spain)*
(First received July 11th, 1989; revised manuscript received October 27th, 1989)

Abscisic acid (ABA) is an important plant hormone involved in several processes of plant growth, and its physiological effects have been investigated[1,2]. A suitable method for the extraction and quantification of endogenous ABA levels should be simple and involve the minimum number of steps, but at the same time be efficient in purifying the extracts[3]. All analyses that involve higher plants require rigorous clean-up procedures because of the high concentration of interfering compounds and the low concentration of ABA[4,5].

We have developed a procedure for the quantification of ABA that involves prepurification of plant extracts with Sep-Pak $C_{18}$ cartridges[4,6–9] followed by passage through an RP-18 guard column and RP-8 and SAX analytical columns. Mass spectrometry was used for peak identification and also to measure the purity of the analyte.

## EXPERIMENTAL

### *Instrumentation*

A Kontron (Zurich, Switzerland) Model LC 620 liquid chromatograph was used to perform gradients, fitted with a Rheodyne (Berkeley, CA, U.S.A.) Model/125 injector with a 250-$\mu$l loop, a Kontron Uvikon 720 spectrophotometric detector, a Kontron Tracer valve-switching module and a Kontron Anacomp 220 microcomputer as a controller and data processor. The tracer switching valves contain four Rheodyne 7010 valves and two low-pressure valves which can be switched from microcomputer by relays.

Three columns were used: a Brownlee RP-18, 10 $\mu$m (35 × 4 mm I.D.) stainless-steel guard column (Brownlee, Mississauga, Ont. Canada), a Tracer C-8, 5 $\mu$m, 300 Å (150 × 4.6 mm I.D.) stainless-steel column (Teknokroma, Sant Cugat del Vallès, Spain) and a Tracer SAX, 5 $\mu$m (250 × 4 mm I.D.) stainless-steel column (Teknokroma). All the columns were operated at room temperature. The detector was set up at 260 nm and 0.1 absorbance $V^{-1}$.

[a] Present address: Serveis Científico-Tècnics, Universitat de Barcelona, Unitat de Cromatografia i Microanàlisi, Martí i Franquès s/n, 08028-Barcelona, Spain

0021-9673/90/$03.50 

The eluents used were: (A) 6.8 m*M* phosphoric acid, (B) methanol and (C) 2 m*M* acetic acid–methanol (60:40, v/v). All of these were degassed by helium flushing. The flow-rate was 1 ml $min^{-1}$.

A Hewlett-Packard HP 5988 A mass spectrometer at 70 eV was used for ABA peak confirmation. The temperature of the interface was 200°C and the temperature of the ion source was 300°C.

*Chemicals and reagents*

Methanol of HPLC grade (Probus, Badalona, Spain), phosphoric acid, acetic acid, 2,6-di-*tert.*-butyl-4-methylphenol (BHT), sodium hydrogencarbonate and (±)-abscisic acid (Fluka, Buchs, Switzerland) were used without further purification Milli-Q water (Millipore, Mulhouse, France) was used to prepare solutions.

*Plant culture*

Plants of *Fatsia japonica* were grown in the experimental fields of the Faculty of Biology (Barcelona University). Maximum photosynthetic photon flux density (PPFD) in the shadehouse was 470 $\mu$mol $m^{-2}$ $s^{-1}$ (maximum natural illumination). The minimum and maximum (daily mean) air temperatures were 18 and 25°C, respectively.

Control plants were watered daily to the container capacity and supplied with a nutrient solution. Other plants were subjected to varying degrees of water stress by withholding water supplies for different periods of time, from one to four days.

Leaves were detached, weighed and immediately frozen in liquid nitrogen and lyophilized before storage at −40°C until ABA extraction.

*Sample preparation*

From 100 to 500 mg (dry weight) of lyophilized and homogenized leaf tissue were shaken in 200 ml of methanol–6.8 m*M* phosphoric acid (80:20, v/v) with 100 mg $l^{-1}$ of 2,6-di-*tert.*-butyl-4-methylphenol as antioxidant at 4°C in the dark for 24 h on a shaker. The homogenate was filtered through an AP Prefilter (Millipore, Bedford, MA, U.S.A.). The remaining fraction was again extracted with 100 ml of methanol–6.8 m*M* phosphoric acid (80:20, v/v) for 4 h under the same conditions until the plant material became colourless. The filtrate was adjusted to pH 8.5 with 0.6 *M* sodium hydrogencarbonate and reduced in a Büchi rotary evaporator at 35°C to an aqueous phase, which phase was frozen at −20°C until the prepurification procedure.

In the prepurification step, the aqueous phase was defrozen, poured into a beaker and the flask was washed with 2 ml of Milli-Q water. The remaining solid suspension was filtered off. The pH was adjusted to 2.6 with concentrated phosphoric acid (8.6 *M*) and loaded with a syringe onto a Sep-Pack $C_{18}$ cartridge, prewetted with 5 ml of methanol and then with 5 ml of water. The cartridge was washed with 5 ml of methanol–6.8 m*M* phosphoric acid (30:70, v/v) to elute the most polar compounds. The cartridge was then washed with 3 ml of methanol–6.8 m*M* phosphoric acid (60:40, v/v) and the eluted fraction was evaporated to dryness in a lyophilizer.

*Determination of abscisic acid*

The dried sample was dissolved in 250 $\mu$l of methanol–6.8 m*M* phosphoric acid (50:50, v/v) and 200 $\mu$l were injected into the high-performance liquid chromato-

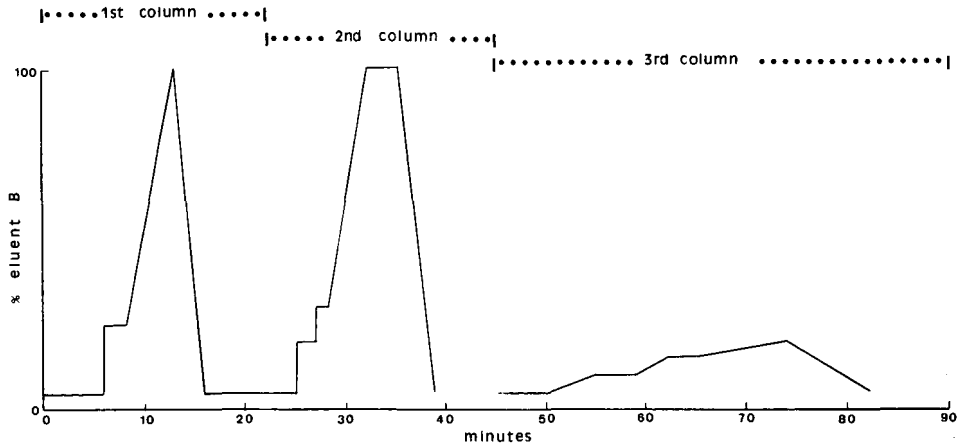



Fig. 1. Gradient scheme relative to eluent B (% methanol) during 90 min of the chromatographic separation that included three different columns: first RP-18, second RP-8 and third SAX.

graphic (HPLC) system for ABA separation and further quantification. The gradient programme included the necessary changes in the valves to turn each column system on or off (Fig. 1).

The eluents for the first (Brownlee RP-18) column were A and B. From time zero (injection) to 6 min, the relative proportion of B was 5% and from 6 to 8 min it was 25%; from 8 to 13 min there was a linear gradient from 25 to 100% B. The eluate from 9 to 10.50 min was introduced automatically at the head of the second (Tracer C-8) column. The eluents for the second colum were also A and B as follows: from 0 to 4 min, 100% A; from 4 to 6 min, 20% B; from 6 to 7 min, 30% B; from 7 to 11 min there was a linear gradient from 30 to 100% B. The eluate from 11 to 13 min was introduced into the head of the third Tracer SAX column. The eluents for this column were C and B as follows: from 0 to 5 min, 5% B; from 5 to 10 min there was a linear gradient from 5 to 10% B; from 10 to 14 min, 10% B; from 14 to 17 min a linear gradient was run from 10 to 15% B; from 17 to 20 min, 15% B; and from 20 to 29 min, 20% B. The ABA fraction was eluted at 5.7 min. The time involved in the whole process was 90 min.

The fraction containing the ABA peak was collected after detection. It was lyophilized and stored at $-20°C$ until mass spectrometric analysis for peak confirmation.

*Calibration graph*

ABA was identified by comparison with the retention time of standards. For the calibration graph, amounts from 42 to 529 ng were used. Standards solutions containing 0.21, 0.24, 0.26, 0.53, 0.80, 1.32, 1.85 and 2.64 ng $\mu l^{-1}$ of ABA in methanol–6.8 m*M* phosphoric acid (1:1, v/v) were prepared and three replicates of each were injected into the HPLC system.

The calibration graph of peak area *vs.* ABA concentration was linear from 42 to 529 ng of ABA standard (correlation coefficient $r = 0.9976$). A calibration run was made daily.

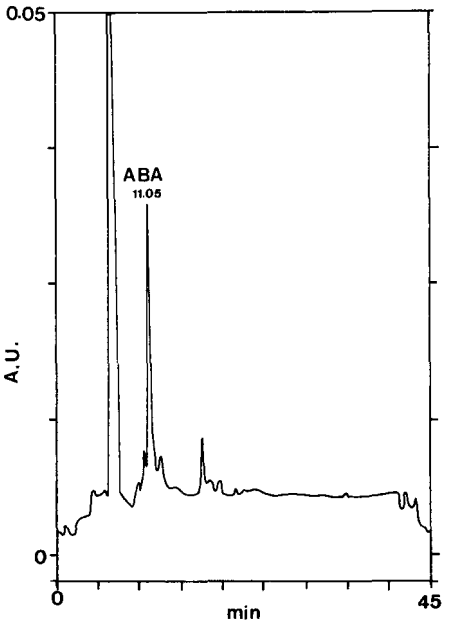



Fig. 2. Chromatography of 0.26 g dry weight of *Fatsia japonica* leaf extract, passed through a Sep-Pak $C_{18}$ cartridge and redissolved in 250 $\mu$l of methanol–6.8 m*M* phosphoric acid (1:1, v/v). Injection volume 200 $\mu$l. The ABA peak was eluted from the SAX column at 5.7 min, which is equivalent to 11.05 min in the second part of the chromatography.

## RESULTS

### *Clean-up procedure*

The method for the prepurification of the crude leaf extract on Sep-Pak $C_{18}$ proved to be a simple and efficient means of removing substances that interfere with subsequent chromatographic steps. The sample and eluent for the clean-up in reversed-phase chromatography had to be acidic for maximum recovery and to reduce tailing. Of the acids tested, phosphoric acid was the most satisfactory in controlling pH. The RP-18 cartridge and automated cut-off are nevertheless necessary in order to avoid damaging the analytical columns.

Chromatographic analyses were performed better by using a sequence of two differents mechanisms. In the first, the RP-8 column separates the components in order of their hydrophobicity, and in the second, an automatic cut-off fraction from the RP-8 eluate is separated by using a strong anion-exchange column.

### *Quantification*

For ABA quantification, an SAX column provides satisfactory resolution and a good separation from interfering substances from the plants. We selected a methanol–2 m*M* acetic acid (60:40, v/v) mobile phase for the SAX column because it gave a good separation of ABA from impurities (Fig. 2). If phosphate is used in the SAX column it does not give a satisfactory performance.

TABLE I

QUANTIFICATION OF ABA LEVELS IN *FATSIA JAPONICA* LEAVES

Different levels of ABA [ng $g^{-1}$ dry weight (D.W.)] in *Fatsia japonica* leaves subjected to varing degrees of water stress from 2 to 4 days. (A) control plants; (B) plants with 2 days of water deficit; (C) plants with 3 days of water deficit; (D) plants with 4 days of water deficit. All plants were sampled at 12 p.m. solar time. Samples of the same plant (A-1, A-2, A-3; B-1, B-2, B-3; C-1, C-2, C-3; D-1, D-2, D-3) showed very similar ABA levels. Also, differences in ABA concentrations in the four treatment groups are obvious.

| *Plant* | *Treatment* | *Sample* | *ABA* (ng $g^{-1}$ D.W.) | $\bar{X}$ (ng $g^{-1}$ D.W.) | *S.D.* (ng $g^{-1}$ D.W.) | *R.S.D.* (%) |
|---|---|---|---|---|---|---|
| A | Control | A-1 | 256.39 | 267.35 | 12.52 | 4.68 |
| | | A-2 | 264.67 | | | |
| | | A-3 | 280.99 | | | |
| B | 2 days stress | B-1 | 929.33 | 922.10 | 22.02 | 2.39 |
| | | B-2 | 939.60 | | | |
| | | B-3 | 897.37 | | | |
| C | 3 days stress | C-1 | 1759.90 | 1780.54 | 137.36 | 7.71 |
| | | C-2 | 1927.05 | | | |
| | | C-3 | 1654.67 | | | |
| D | 4 days stress | D-1 | 3031.54 | 3291.95 | 240.48 | 7.31 |
| | | D-2 | 3338.70 | | | |
| | | D-3 | 3505.63 | | | |

The precision is good because several injections of the same sample into the HPLC system gave the same result, as did analyses of different samples that had been subjected to the same water stress conditions (Table I). Further the reproducibility is very good. The relative standard deviation (R.S.D.)[10] was 6.22% with a range from 5.26% to 7.18% ($n$ = 21) over nine days.

Mass spectrometry was used for peak confirmation. After separation of ABA from other plant substances and collection of this fraction, mass spectrometry gave satisfactory confirmation of the peak substance (Figs. 3 and 4).

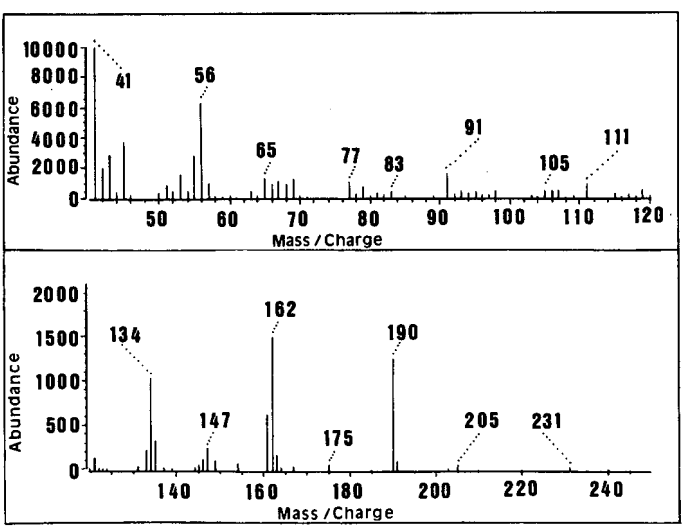



Fig. 3. Electron impact mass fragmentation patterns of ABA standard.

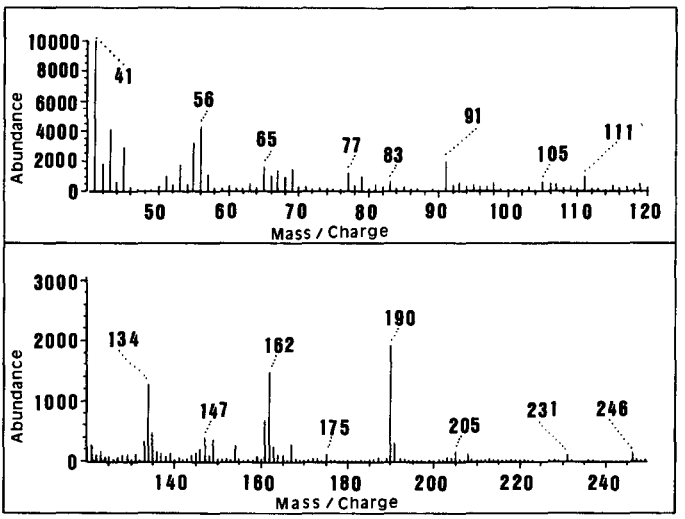



Fig. 4. Electron impact mass fragmentation of ten peaks collected from the HPLC column after ten injections of *Fatsia japonica* leaf extracts.

*Recovery and limit of quantitation*

We determined the recovery by adding a solution of ABA standard to leaf extracts. The recovery was 62-69 ± 16.89% (standard error).

The limits of quantification for ABA standard and for endogenous ABA were different. For ABA standard the limit was 159 pg $\mu l^{-1}$, whereas for endogenous ABA the limit was *ca.* 53 ng $g^{-1}$ dry weight, calculated for a peak signal-to-noise ratio of 2. This is because baseline resolution is not achieved completely in the region of the chromatogram where ABA elutes.

## DISCUSSION

This method has good precision, good recovery and high reproducibility. It should be noted that the important criterion of a method is the extent of variation that occurs from sample to sample; the recovery do not reflect the quality of the method[10,11]. This is an important consideration when it is necessary to measure ABA levels during daily stress cycles and subsequent recovery. When one of these processes is to be studied, a large amount of sample is collected for further ABA analyses. It is then necessary to have a simple method in order to carry out large numbers of analyses and avoid handling of samples.

The total recovery of ABA with the present method was 62.69%, which is similar to those reported by other investigators[8,12–14].

The R.S.D. for the entire system was 6.22%, which is similar to the value reported by Bousquet *et al.*[7] and comparable to the valve of 10% reported by Kling *et al.*[10] for indoleacetic acid (IAA). We cannot compare the reproducibility of our method with that of other techniques because many workers did not report the R.S.D.

Previously it was necessary to work with a large amount of leaves per sample

(1–20 g dry weight), and the extraction process was tedious and the purification step very difficult. Only small amounts of leaf material per sample are needed in the present method and we can also use the same leaf for other biochemical and ecophysiological analyses. Further, small amounts of plant material lead to less extensive chromatographic interferences.

In contrast to Kling *et al.*[10], in our method an SAX column provides a good means for quantification of ABA. There is no variability of retention time, but the life of the column is shorter than that of other types of column. With a SAX column we could determine ABA in 190 leaf extracts and 82 standards before renewal of the packing material became necessary.

We consider the method described here to be selective for ABA because the putative ABA peak is symmetrical without shoulders, the detector was set for the wavelength of maximum absorption of ABA and the identity of the presumed ABA peak from leaf extracts of *Fatsia japonica* recorder by the detector was confirmed by mass spectrometry. Comparison of the mass spectra of the ABA from the samples with an ABA standard showed them to be identical.

This method, in its present form, is valid only for the determination of ABA; no other plant hormones such as IAA can be determined in this way because, taking into consideration results in the literature and our experience, the plant material that we use is lyophilized and not fresh, when the aqueous phase after extraction is dried *in vacuo* IAA can be lost by sublimation and the measurement of UV absorbance is much less specific than fluorescence for IAA.

We believe that this method can be used in a wide range of experiments which require the quantification of ABA. Routine analyses of ABA may be performed in the near future using this method.

ACKNOWLEDGEMENTS

The authors thank Mr. Carles Celma of the Department de Quimica Orgànica de la Universitat de Barcelona for technical support with the mass spectrometric analyses. This work was supported by a CIRIT grant (Generalitat de Catalunya).

REFERENCES

1 S. J. Neil and R. Horgan, in L. Rivier and A. Crozier (Editors), *The Principles and Practice of Plant Hormone Analysis*, Vol. I, Academic Press, London, 1987, Ch. 3, p. 111.
2 J. A. D. Zeevaart and R. A. Creelman, *Annu. Rev. Plant Physiol. Mol. Biol., 39 (1988) 439.*
3 *R. C. Durley, T. Kannangara and G. M. Simpson, Can. J. Bot.*, 56 (1978) 157.
4 S. M. Norman, V. P. Maie and L. C. Echols, *J. Liq. Chromatogr.*, 5 (1982) 81.
5 T. Kannangara, G. M. Simpson, K. Rajkumar and B. D. Murphy, *J. Chromatogr.*, 283 (1984) 425.
6 G. T. Vaughan and B. V. Milborrow, *J. Chromatogr.*, 336 (1984) 221.
7 E. Bousquet, N. A. Santagati and G. Romeo, *J. Chromatogr.*, 354 (1986) 503.
8 G. Guinn, D. L. Brummett and R. C. Beier, *Plant Physiol.*, 81 (1986) 997.
9 E. Vermeer, E. Knegt and J. Bruinsma, *J. Chromatogr.*, 404 (1987) 346.
10 G. J. Kling, L. M. Perkins, P. E. Cappiello and B. A. Eisenberg, *J. Chromatogr.*, 407 (1987) 377.
11 A. Majcherczyk, L. Rakoczy and A. Hüttermann, *J. Chromatogr.*, 357 (1986) 399.
12 M. Hein, M. L. Brenner and W. A. Brun, *Plant Physiol.*, 76 (1984) 951.
13 J. A. D. Zeevaart, *Plant Physiol.*, 71 (1983) 477.
14 K. C. Hall, S. J. Chapman and D. G. Christian, *J. Sci. Food Agric.*, 37 (1986) 219.

*Journal of Chromatography*, 503 (1990) 466–470
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 215

# Note

## Analysis of nitrogen dioxide in ambient air by ion-exclusion chromatography with electrochemical detection

HIE-JOON KIM
*Kim & Associates, 33 Pleasant Street, Wayland, MA 01778 (U.S.A.)*
(First received September 21st, 1989; revised manuscript received December 12th, 1989)

Nitrogen dioxide ($NO_2$) is an important air pollutant and a precursor of acid rain. It is produced from nitrogen oxide (NO) in the atmosphere and plays a key role in the occurrence of smog[1]. With increasing global efforts to reduce emission of acid rain precursors such as sulfur dioxide ($SO_2$) and nitrogen oxides ($NO_x$), it is desirable to have a sensitive and convenient method for monitoring $NO_2$ present at ppb[a] concentrations in the ambient air.

Two common methods for $NO_2$ in ambient air are the chemiluminescence method[2] and the Saltzman method[3]. These and other instrumental methods such as laser absorption spectrometry[4] can measure $NO_2$ at ppb concentrations but are inconvenient for measurements at remote sites not covered by the air monitoring stations.

Trapping $NO_2$ in the air on a solid cartridge and determining the trapped $NO_2$ at a central laboratory is an economical and logistically sound alternative. In 1958, Jacobs and Hochheiser[5] described trapping $NO_2$ in 0.1 *M* sodium hydroxide solution and analyzing the resulting nitrite ($NO_2^-$) by diazotization–coupling reaction. Subsequent work involved trapping $NO_2$ on solid sampling devices followed by diazotization–coupling reaction[6,7], ion-exchange chromatography with conductivity detection[8–12], or reversed-phase high-performance liquid chromatography with UV detection[13].

Recently, we demonstrated that nitrite can be determined with an extremely high sensitivity and specificity using ion-exclusion chromatography with electrochemical detection[14]. It is the purpose of this paper to show that $NO_2$ in the ambient air at ppb or sub-ppb concentrations can be conveniently trapped and determined with a high sensitivity using ion-exclusion chromatography with electrochemical detection.

## EXPERIMENTAL

### *Air sampling*

Triethanolamine–sodium hydroxide-coated cartridge was prepared with a slight modification of the published procedure[11]. Maxi-Clean $C_{18}$ cartridge (300-mg size,

[a] Throughout this article the American billion ($10^9$) is meant.

0021-9673/90/$03.50 

Alltech, Deerfield, IL, U.S.A.) was washed by passing 3 ml methanol and 5 ml deionized water. Then 5 ml solution of 2% TEA (Baker, Phillipsburg, NJ, U.S.A.) and 1% sodium hydroxide in 50% aqueous methanol was passed through the cartridge. The cartridge was dried under infrared lamp for 2 h and both ends were sealed with Parafilm to protect from air until use.

LaMotte Model BD air sampling pump and Model LD adjustable flow meter (LaMotte, Chestertown, MD, U.S.A.) were used for sampling air. Outdoor sampling was facilitated by battery operation. Ambient air samples were collected at 1.0 l/min flow-rate for every 3 h about 1 ft. above ground in Wayland, MA, U.S.A. (a suburb about 15 miles west of Boston). Indoor air was sampled for 30 min at 1.0 l/min.

*Apparatus*

A Wescan Model 361 sulfite analyzer (Deerfield, IL, U.S.A.) was used for analysis of $NO_2^-$. It was equipped with an anion-exclusion Ion-Guard cartridge, anion-exclusion/HS column (sulfonated polystyrene–divinylbenzene; 100 × 4.6 mm I.D.), Rheodyne injector with a 50-$\mu$l sample loop, a Wescan Model 271 electrochemical detector with a platinum working electrode and a Ag/AgCl reference electrode, and a computing integrator (Spectra-Physics 4290, San Jose, CA, U.S.A.). The electrode surface was occasionally cleaned by manually setting the voltage at $-1.0$ V for several min and then at $+1.8$ V for another several min before reequilibrating the system at $+1.0$ V. The sulfite analyzer could also deliver a similar electrode cleaning pulse sequence, with a shorter duration, after each sample injection.

*Analysis*

Two aliquots of 4 ml 0.1 *M* sodium hydroxide solution were passed successively through the cartridge and the eluting solutions were injected directly into the chromatograph. A standard solution containing 0.1–0.5 ppm $NO_2^-$ was injected next to the sample and the signal intensity was compared with the sample. The eluent was a 5 m*M* sulfuric acid solution degassed under vacuum. The flow-rate was 0.8 ml/min. The detector voltage was $+1.0$ V *vs.* Ag/AgCl reference electrode[14].

*Calculation*

A stoichiometric factor of 0.72 was used for conversion of $NO_2$ to $NO_2^-$ (ref. 3). At the average ambient temperature of 25°C, 1 $\mu$g $NO_2/m^3$ corresponds to 0.532 ppb (v/v). Therefore, the $NO_2$ concentration in ppb derived from nitrite ion in the 4 ml eluting solution is given by:

$$NO_2 \text{ (ppb, v/v)} = \frac{(\text{peak height for sample})(\text{ppm in standard} \times 4)(0.532)}{(\text{peak height for standard})(\text{sampling time in min})(10^{-3})(0.72)}$$

The contribution from the second 4 ml eluting solution was combined with the first 4 ml to yield the $NO_2$ concentration in the air.

## RESULTS AND DISCUSSION

In 1970, Robinson and Robbins[15] considered the global atmospheric nitrogen cycle and estimated the ambient concentration of $NO_2$ on land to be 4 ppb. Schiff *et*

*al.*[4] observed a variation between 0.2 and 2.8 ppb during a 24-h period at a rural site. The U.S. Environmental Protection Agency (EPA) established the maximum allowable increase in ambient $NO_2$ concentration in Class I areas as 2.5 $\mu g/m^3$ (1.3 ppb)[16]. These figures suggest that an analytical method with a sub-ppb detection limit is needed to monitor the ambient $NO_2$ concentration. Commercially available chemiluminescence analyzers can detect sub-ppb levels of $NO_2$ in real time. Nevertheless, such *in situ* analyzers are expensive and not suitable for measurements at remote locations not covered by routine monitoring.

A logistically sound alternative is to collect air samples using an inexpensive sampling device and analyze the trapped $NO_2$ at a central laboratory. In this approach, the detection limit normally dictated by the sensitivity of the analytical method can be decreased by increasing the air sample volume. Conversely, a smaller volume of air will be required to achieve the same detection limit if a more sensitive analytical technique is used. For example, to achieve 1 ppb detection limit, 1200 l air sample is needed using diazotization–coupling method[6] and 250 l is needed by ion-exchange chromatography with conductivity detection[10].

It is well known that amperometric detection offers higher sensitivity than conductivity detection. A detection limit of about 1 ppb $NO_2^-$ by ion-exchange chromatography with amperometric detection[17,18] has been reported. Under optimal conditions, we obtained a detection limit of 0.1 ppb $NO_2^-$ in solution[14]. With such

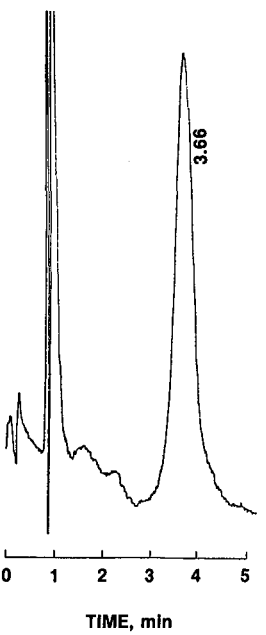



Fig. 1. Chromatogram of nitrite corresponding to 0.19 ppm in 4 ml 0.1 *M* sodium hydroxide eluting solution. Ambient air (180 l) was collected on a $C_{18}$ cartridge treated with triethanolamine–sodium hydroxide and the cartridge was eluted with 4 ml eluting solution. Nitrite in the solution was determined using a Wescan Model 361 Sulfite Analyzer with an anion exclusion-HS column, a Pt working electrode set at +1.0 V *vs.* Ag/AgCl reference electrode. Eluent, 5 m*M* sulfuric acid; flow-rate, 0.8 ml/min. Injection volume, 50 $\mu$m. The maximum current for the nitrite peak at 3.66 min was 58 nA.

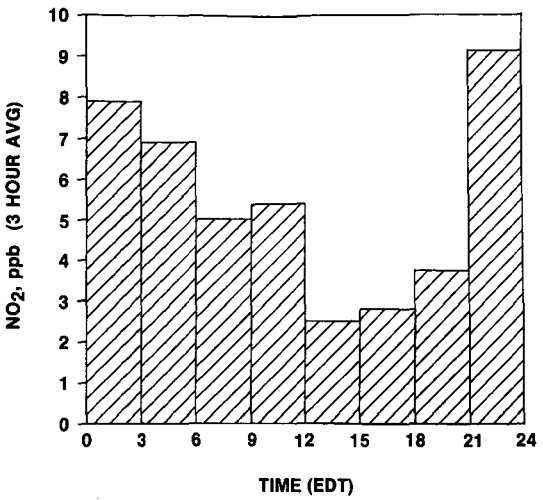



Fig. 2. Variation of 3 h average (AVG) $NO_2$ concentration in Wayland, MA, U.S.A. observed by ion-exclusion chromatography with electrochemical detection on July 23, 1989. EDT = Eastern Daylight Time.

a sensitivity, one can detect 1 ppb $NO_2$ in air with about 0.3 l sample, which represents a tremendous improvement in sensitivity over the previous methods[6–13].

In most cases, it is desirable to obtain an average $NO_2$ concentration over many hours. For example, EPA requires that State and Local Air Monitoring Stations (SLAMS) determine 24 h average when manual methods are used[19]. Fig. 1 shows a nitrite peak (0.19 ppm) at 3.66 min resulting from sampling 180 l ambient air in Wayland, MA, U.S.A., on a cartridge and eluting with 4 ml 0.1 *M* sodium hydroxide solution. Using the formula under *Calculation*, the $NO_2^-$ concentration was converted to 3.1 ppb $NO_2$ in the air. The Saltzman factor of 0.72 was used for the equivalence of $NO_2$ to $NO_2^-$ (ref. 3). Other reported values for the equivalence are 0.76 by Scaringelli *et al.*[20], 0.85 by Levaggi *et al.*[6], 0.63 by Blacker[7], 0.64 by Vinjamoori and Ling[8], and 0.83 by Nishikawa *et al.*[10]. $NO_2^-$ corresponding to 0.5 ppb $NO_2$ in air was observed in the second 4 ml eluting solution. Therefore, the total $NO_2$ concentration in the air was 3.6 ppb. No $NO_2^-$ was observed from a control cartridge.

$NO_2$ concentration of indoor air was measured similarly with 30 l air samples. 14.9 ppb $NO_2$ was observed near a gas burner in the basement. 3.7 ppb was observed in the living room. Others reported higher values in both living area and the kitchen with a gas stove[12,13].

Fig. 2 shows the variation of 3 h average ambient $NO_2$ concentration on July 23, 1989. The $NO_2$ concentration reached a maximum of 9.1 ppb around midnight and decreased gradually due to oxidation by ozone[4]. It increased slightly between 9 a.m. and noon possibly due to emission from the morning traffic. During the day, it decreased to a minimum of 2.5 ppb by photochemical reactions[4] and increased after sunset. A similar pattern was observed by Schiff *et al.*[4]. The daily minimum of 2.5 ppb is higher than about 0.2 ppb observed at Cold Creek, Canada, a clean rural site. The maximum of 9.1 ppb is well below the maximum permissible concentration of 0.05 ppm $NO_2$ (annual arithmetic mean).

These data suggest that the trapping of $NO_2$ on a solid cartridge followed by ion-exclusion chromatography with electrochemical detection may be a sensitive and convenient technique for measuring time-average $NO_2$ concentration at ppb levels in the ambient air. A 0.05 ppm nitrite peak is expected from 24 h sampling of air containing 0.1 ppb $NO_2$. The intensity of the 0.19 ppm $NO_2^-$ peak in Fig. 1 suggests that this can be easily achieved. The high sensitivity suggests that a passive sampling device can be used to detect $NO_2$ at ppb concentrations with a short exposure. Mulik *et al.*[12] showed that 13 ppb $NO_2$ can be determined with a passive sampling device with 1 h exposure using ion-exchange chromatography and conductivity detection. The sensitivity of the passive sampling method can be significantly improved using the present method. The convenience of sampling and the sensitivity of the present method will be particularly useful for investigating the vertical concentration profile of the pollutant, which is critical for understanding the long range transport of this acid rain precursor[21].

REFERENCES

1 E. I. Shaheen, *Environmental Pollution: Awareness and Control*, Engineering Technology, Mahomet, IL, 1974, p. 46.
2 A. Fontijn, A. J. Sabadell and R. J. Ronco, *Anal. Chem.*, 42 (1970) 575.
3 B. E. Saltzman, *Anal. Chem.*, 26 (1954) 1949.
4 H. I. Schiff, G. W. Harris and G. I. Mackay, in R. W. Johnson and G. E. Gordon (Editors), *The Chemistry of Acid Rain* (*ACS Symposium Series*, No. 349), American Chemical Society, Washington, DC, 1987, p. 274.
5 M. B. Jacobs and S. Hochheiser, *Anal. Chem.*, 30 (1958) 426.
6 D. A. Levaggi, W. Siu and M. Feldstein, *J. Air Pollut. Control Assoc.*, 23 (1973) 30.
7 J. H. Blacker, *Am. Ind. Hyg. Assoc. J.*, 34 (1973) 390.
8 D. V. Vinjamoori and C.-S. Ling, *Anal. Chem.*, 53 (1981) 1689.
9 D. Brocco and R. Tappa, *J. Chromatogr.*, 367 (1986) 240.
10 Y. Nishikawa, K. Taguchi, Y. Tsujino and K. Kuwata, *J. Chromatogr.*, 370 (1986) 121.
11 Y. Nishikawa and K. Taguchi, *J. Chromatogr.*, 396 (1987) 251.
12 J. D. Mulik, R. G. Lewis, W. A. McClenny and D. D. Williams, *Anal. Chem.*, 61 (1989) 187.
13 F. Lipari, *Anal. Chem.*, 56 (1984) 1820.
14 H.-J. Kim and Y.-K. Kim, *Anal. Chem.*, 61 (1989) 1485.
15 E. Robinson and R. C. Robbins, *J. Air Pollut. Control Assoc.*, 20 (1970) 303.
16 EPA, Federal Register, 53 (1988) 40656.
17 V. Kordorouba and M. Pelletier, *Mitt. Geb. Lebensmittelunters. Hyg.*, 79 (1988) 90.
18 P. Pastore, I. Lavagnini, A. Boaretto and F. Magno, *J. Chromatogr.*, 475 (1989) 331.
19 EPA, *Code of Federal Regulations*, 40, Ch. 1, Pt. 58, Subpart B, Office of the Federal Register, National Archives and Records Administration, Washington, DC, 1988, p. 130.
20 F. P. Scaringelli, E. Rosenberg and K. A. Rehme, *Environ. Sci. Technol.*, 4 (1970) 924.
21 G. E. Gordon, in R. W. Johnson and G. E. Gordon (Editors), *The Chemistry of Acid Rain*, (*ACS Symposium Series*, No. 349), American Chemical Society, Washington, DC, 1987, p. 4.

*Journal of Chromatography*, 503 (1990) 471–472
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 289

# Book Review

*Principles and practice of chromatography*, by B. Ravindranath, Ellis Horwood, Chichester, 1989, 497 pp., price £ 58.50, ISBN 0-7458-0296-6.

In contrast to earlier texts involving chromatography in the *Ellis Horwood Series in Analytical Chemistry* this book provides an overview of the subject and as such should appeal to a wider cross-section of the scientific community than its predecessors. Furthermore, the author's decision to place a strong emphasis on the chemical principles underlying the various chromatographic methods is to be applauded. For each technique considered the theory is explained simply, yet thoroughly, in an easy-to-follow style, a fact that commends adoption of the book as a standard text for undergraduate analytical chemistry courses. The book also should prove to be equally useful to post-graduate research students and to scientists working in industry.

The book consists of four parts devoted to basic principles, gas chromatography, liquid chromatography and analytical applications, respectively.

Part 1 (basic principles) consists of two chapters. The first features an overview of classical separation processes, such as distillation, solvent extraction and counter-current distribution, together with contemporary chromatographic techniques. Also included is a review of the historical development of chromatography (essential reading for the Ph.D. student about to write a thesis) and useful sections on the nomenclature and the literature of chromatography. The next chapter deals with the theoretical concepts with a strong bias towards zone dispersion processes. Here it is worth noting the derivations of the various plate height equations.

Gas chromatography is considered in Part 2, which consists of three chapters dealing with sampling, separation systems and detectors, respectively. After a brief introduction to the gas chromatograph the author immediately turns to optimization, which unfortunately is treated in a rather cursory manner. This is then followed by a discussion of sample handling, including derivatization, headspace and pyrolysis techniques, together with a reasonably comprehensive description of injection systems, including devices for use with packed and capillary columns. The next chapter deals with the carrier gas, liquid phases and column packings. Here details are given of the analytical applications of a wide range of materials, including the less familiar phases such as steam-containing carrier gases, organic salts, modified cyclodextrins and liquid crystalline materials. Although the scope of the chapter is difficult to fault, many readers will be disappointed to find little discussion of selectivity and its use in analysis. The third chapter deals essentially with those devices familiar to the majority of analysts. The decision by the author to omit ion-trap devices from the treatment of modern gas chromatography–mass spectrometry is regrettable.

Over a third of the book is devoted to Part 3 which consists of three chapters dealing with the principles and methods of liquid chromatography, instrumentation techniques and planar chromatography, respectively. The first chapter starts with

a consideration of classical column chromatography and leads to a substantial discussion of size-exclusion chromatography. This is followed by brief accounts of the principles of the various forms of field-flow fractionation and partition systems, including droplet counter-current chromatography. In a similar vein the treatment of adsorption systems is rather weak, on the other hand the accounts of ion-exchange and affinity chromatography are excellent. The second chapter consists of a reasonably comprehensive treatment of the instrumentation and techniques of high-performance liquid chromatography. Also included in this chapter is a useful account of approaches to method development. The final chapter is devoted to contemporary planar chromatography. On reading this part of the book one gains the impression that the author has been too ambitious in trying to cover all aspects of liquid chromatography. The result is a rather cursory treatment without real depth; good for the absolute beginner but not too much use to the established worker.

A review of the applications of chromatography is presented in Part 4. Although a very readable and interesting section of the book it is not particularly informative. Much of the material could have been more profitably included with the earlier chapters dealing with specific techniques.

The book is well written and nicely presented. Each chapter is logically structured and is supported by appropriate references. Although it may not appeal to the specialist it could serve the needs of analysts who from time to time need to use separation methods.

*Hatfield (U.K.)* M. B. EVANS

*Journal of Chromatography*, 503 (1990) 473–474
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 288

# Book Review

*Inverse GC characterization of polymers and other materials*, edited by D. R. Lloyd, T. C. Ward and H. P. Schreiber, American Chemical Society, Washington, DC, 1989, xii + 331 pp., price US$ 69.95 (U.S.A. and Canada), US$ 83.95 (rest of world), ISBN 0-8412-1610-X.

This work forms No. 391 of the *ACS Symposium Series* and is based on the papers presented at a symposium held as part of the 95th National Meeting of the ACS held in Toronto in June, 1988.

Consisting of 21 chapters the work commences with an overview and is then subdivided into six groups, namely Methodology and instrumentation (3 chapters), Sorption and diffusion in polymers (4 chapters), Polymer blend characterization (4 chapters), Surface and interface considerations (6 chapters), Analytical and Special application (each 2 chapters).

The overview indicates that inverse gas chromatography, although developed in 1967, found almost negligible use in the first decade but the second decade exhibited tremendous growth forming about 30% of all gas chromatography publications. The potential of this and other non-analytical techniques has been suggested since the early days of chromatography but has found little application. The relationship between retention, both as specific retention volumes and the practical retention indices, and thermodynamic parameters gives the techniques added emphasis, and the bulk of the chapter, therefore introduces these groups of contributions.

The second chapter indicates briefly that standard gas chromatographs are suitable for use. The two following methodical contributions detail the determination of polymer structure and the computer simulation of the elution of the probes used.

Sorption and diffusion effects are first illustrated by the calculation of solubility parameters using the method of Guillet and Di Paola-Baranji, the limitations of the procedure being discussed. The other contributions in the grouping consider the effect on polymers of Gas and vapour adsorption, water sorption and water vapour diffusion and solute diffusion.

Polymer blends are of increasing commercial importance and the thermodynamics theory, free energy and interaction polymers are each outlined.

Surface and interfacial characterization considers carbon, plasma-treated carbon and glass fibres and interactions between carbon fibres and epoxy resins and fibre–matrix adhesion and the analysis of solid surface modifications.

The chapters are largely mathematical as are the application chapters. The characterization of stationary phase siloxanes using the retention of aliphatic and aromatic solutes demonstrates that the slopes for the *n*-alkanes are particularly sensitive to the aromatic content of the solvents. The characterization of sorbent materials utilises trace pulse chromatography based on the work of Parcher. The practical application of inverse gas chromatography is demonstrated with the examination of coals.

The final chapter presents a modification of the conventional inverse gas chromatography approach. A modified frontal procedure was developed avoiding assumptions of equilibrium to allow studies of interaction parameters of phases possessing both variable entropic and enthalpic relations.

Although the number of chapters is quite large, all chapters are brief and concise to provide a work of moderate size. The authors are largely major workers who have contributed to the development of the technique providing extensive and timely bibliographies.

The work is particularly recommended to workers in inverse chromatography although it is apparent that a background in physical chemistry is almost a prerequisite to inverse gas chromatography, or at least to its theoretical basis.

*Kensington (Australia)* JOHN K. HAKEN

*Journal of Chromatography*, 503 (1990) 475
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 212

# Book Review

*Supercritical fluid extraction and chromatography (ACS Symposium Series*, No. 366), edited by B. A. Charpentier and M. R. Sevenants, American Chemical Society, Washington, DC, 1988, IX + 253 pp., price US$ 99.95, ISBN 0-8412-1469-7.

This book developed from a symposium sponsored by the Division of Agricultural and Food Chemistry at the *193rd Meeting of the American Chemical Society, Denver, CO, 1987*. The first chapter gives an overview of the physical chemistry of supercritical fluids. This chapter is quite distinct from the otherwise practical approach of the book and gives the appearance of having been written for fellow physical chemists. The following six chapters deal with extraction with supercritical fluids. Chapter 2 gives a critical review of the events during the last decade, mainly in industrial processing, that created earlier and recent interest in supercritical fluid extraction (SFE). In Chapter 3 analytical methodologies for SFE are discussed and in Chapters 4–6 applications with vegetable oils, fish oils and essential oils are described. The steps to develop a commercial processing plant are analysed in Chapter 7.

The second part of the book deals with supercritical fluid chromatography (SFC). Chapters 8 and 10 present examples of applications in the food industry, Chapter 9 discusses retention processes in SFC and the use of solvatochromic methods for such studies and the last three chapters, 11–13, deal with the hyphenated techniques SFC–mass spectrometry and SFC–Fourier transform infrared spectroscopy.

The book suffers from a lack of homogeneity which reflects the symposium on which it is based. Particularly the chromatographic section is thin. Combining a dash of theory with food-related applications is not likely to give a satisfactory result for the reader who already is familiar with both SFE and SFC. This weakness, however, is also the strength of the book. Readers who need to familiarize themselves with supercritical fluids, particularly with SFE, are now given an opportunity to get a first taste of the subject, or rather the first flavour of the food.

*Oslo (Norway)* TYGE GREIBROKK

## Author Index

## Erratum

*J. Chromatogr.*, 499 (1990) 291–304.

Page 301, 10th text line should read "surface was studied in the static adsorption experiment summarized in Table".

## PUBLICATION SCHEDULE FOR 1990

*Journal of Chromatography and Journal of Chromatography, Biomedical Applications*

| MONTH | J | F | M | A | M | |
|---|---|---|---|---|---|---|
| Journal of Chromatography | 498/1<br>498/2<br>499 | 500<br>502/1 | 502/2<br>503/1<br>503/2<br>504/1 | 504/2<br>505/1 | 505/2<br>506<br>507 | The publication schedule for further issues will be published later |
| Cumulative Indexes, Vols. 451–500 | | 501 | | | | |
| Bibliography Section | | 524/1 | | 524/2 | | |
| Biomedical Applications | 525/1 | 525/2 | 526/1 | 526/2<br>527/1 | 527/2 | |

## INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 478, pp. 453–456. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

**Types of Contributions.** The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Notes, Review articles and Letters to the Editor. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed six printed pages. Letters to the Editor can comment on (parts of) previously published articles, or they can report minor technical improvements of previously published procedures; they should preferably not exceed two printed pages. For review articles, see inside front cover under Submission of Papers.

**Submission.** Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

**Manuscripts.** Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

**Introduction.** Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

**Summary.** Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Notes and Letters to the Editor are published without a summary.)

**Illustrations.** The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

**References.** References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

**Dispatch. Before sending the manuscript to the Editor please check that the envelope contains three copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.**

**Proofs.** One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

**Reprints.** Fifty reprints of Full-length papers, Notes and Letters to the Editor will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

**Advertisements.** Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.

# Analytical Artifacts

## GC, MS, HPLC, TLC and PC

*by* **B.S. MIDDLEDITCH,** *Dept. of Biochemical and Biophysical Sciences, University of Houston, Houston, TX, USA*

**(Journal of Chromatography Library, 44)**

This encyclopaedic catalogue of the pitfalls and problems that all analysts encounter in their work is destined to spend more time on the analyst's workbench than on a library shelf. The author has dedicated the book to "the innumerable scientists who made mistakes, used impure chemicals and solvents, suffered the consequences of unanticipated side-reactions, and were otherwise exposed to mayhem yet were too embarrassed to publish their findings".

Traditionally, the mass spectroscopist or gas chromatographer learnt his trade by participating in a 4-6 year apprenticeship as graduate student and post-doctoral researcher. Generally, no formal training was provided on the things that go wrong, but this information was accumulated by sharing in the experiences of colleagues. Nowadays, many novice scientists simply purchase a computerized instrument, plug it in, and use it. Much time can be wasted in studying and resolving problems due to artifacts and there is also a strong possibility that artifacts will not be recognized as such. For example, most analysts realize that they should use glass rather than plastic containers; but few of them would anticipate the possibility of plasticizer residues on glassware washed using detergent from a plastic bottle.

This book is an easy-to-use compendium of problems encountered when using various commonly used analytical techniques. Emphasis is on impurities, by-products, contaminants and other artifacts. A separate entry is provided for each artifact. For specific chemicals, this entry provides the common name, mass spectrum, gas chromatographic data, CAS name and registry number, synonyms and a narrative discussion. More than 1100 entries are included. Mass spectral data are indexed in a 6-peak index (molecular ion, base peak, second peak, third peak) and there are also formula, author and subject indexes. An extensive bibliography contains complete literature citations.

The book is designed to be *used*. It will not only allow experienced analysts to profit from the mistakes of others, but it will also be invaluable to other scientists who use analytical instruments in their work.

*1989 xxiv + 1028 pages*
*US$ 241.50 / Dfl. 495.00*
*ISBN 0-444-87158-6*

**ELSEVIER SCIENCE PUBLISHERS**

**P.O. Box 211, 1000 AE Amsterdam, The Netherlands**
**P.O. Box 882, Madison Square Station, New York, NY 10159, USA**