ISSN 0021-9673

UBLISHED
VEEKLY

VOL. **247** NO. **2** OCTOBER 1, 1982
THIS ISSUE COMPLETES VOL. 247

OURNAL OF

CHROMATOGRAPHY

NTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

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 (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews,* reviews on all aspects of chromatography, electrophoresis and related methods are published.

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 and will appear in *Chromatographic Reviews* or *Biomedical Applications.* 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 Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.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 Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 221–230, 231–240* and *241–250*) has 25 volumes in 1982. The subscription prices for 1982 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 221–230, 231–240 and 241 250*) + *Biomed. Appl.* (Vols. 227–251):
Dfl. 3625.00 plus Dfl. 500.00 (postage) (total *ca.* US$ 1650.00)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 231–240* and *241–250*) only (Vols. 234–251):
Dfl. 2826.00 plus Dfl. 360.00 (postage) (total *ca.* US$ 1274.50)

Biomed. Appl. (incl. *Cum. Indexes Vols. 221–230*) only (Vols 227–233):
Dfl. 1050.00 plus Dfl. 140.00 (postage) (total *ca.* US$ 476.00).

Journals are automatically sent by airmail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand, with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 226) are available at Dfl. 173.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier Science Publishing Company Inc., Journal Information Center, 52 Vanderbilt Avenue, New York, NY 10017. Tel: (212) 867-9040.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index.

See page 3 of cover for Publication Schedule, Information for Authors, and information on the News Section and Advertisements.

Submission of an article for publication implies the transfer of the copyright from the author(s) to the publisher and entails the authors' irrevocable and exclusive authorization of the publisher to collect any sums or considerations for copying or reproduction payable by third parties (as mentioned in article 17 paragraph 2 of the Dutch Copyright Act of 1912 and in the Royal Decree of June 20, 1974 (S. 351) pursuant to article 16 b of the Dutch Copyright Act of 1912) and/or to act in or out of Court in connection therewith.

Special regulations for readers in the U.S.A. This journal has been registered with the Copyright Clearance Center, Inc. Consent is given for copying of articles for personal or internal use, or for the personal use of specific clients. This consent is given on the condition that the copier pays through the Center the per-copy fee stated in the code on the first page of each article for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. The appropriate fee should be forwarded with a copy of the first page of the article to the Copyright Clearance Center, Inc., 21 Congress Street, Salem, MA 01970, U.S.A. If no code appears in an article, the author has not given broad consent to copy and permission to copy must be obtained directly from the author. All articles published prior to 1980 may be copied for a per-copy fee of US$ 2.25, also payable through the Center. This consent does not extend to other kinds of copying, such as for general distribution, resale, advertising and promotion purposes, or for creating new collective works. Special written permission must be obtained from the publisher for such copying.

Special regulations for authors in the U.S.A. Upon acceptance of an article by the journal, the author(s) will be asked to transfer copyright of the article to the publisher. This transfer will ensure the widest possible dissemination of information under the U.S. Copyright Law.

Printed in The Netherlands

VOL. 247, NO. 2 JOURNAL OF CHROMATOGRAPHY OCTOBER 1, 1982

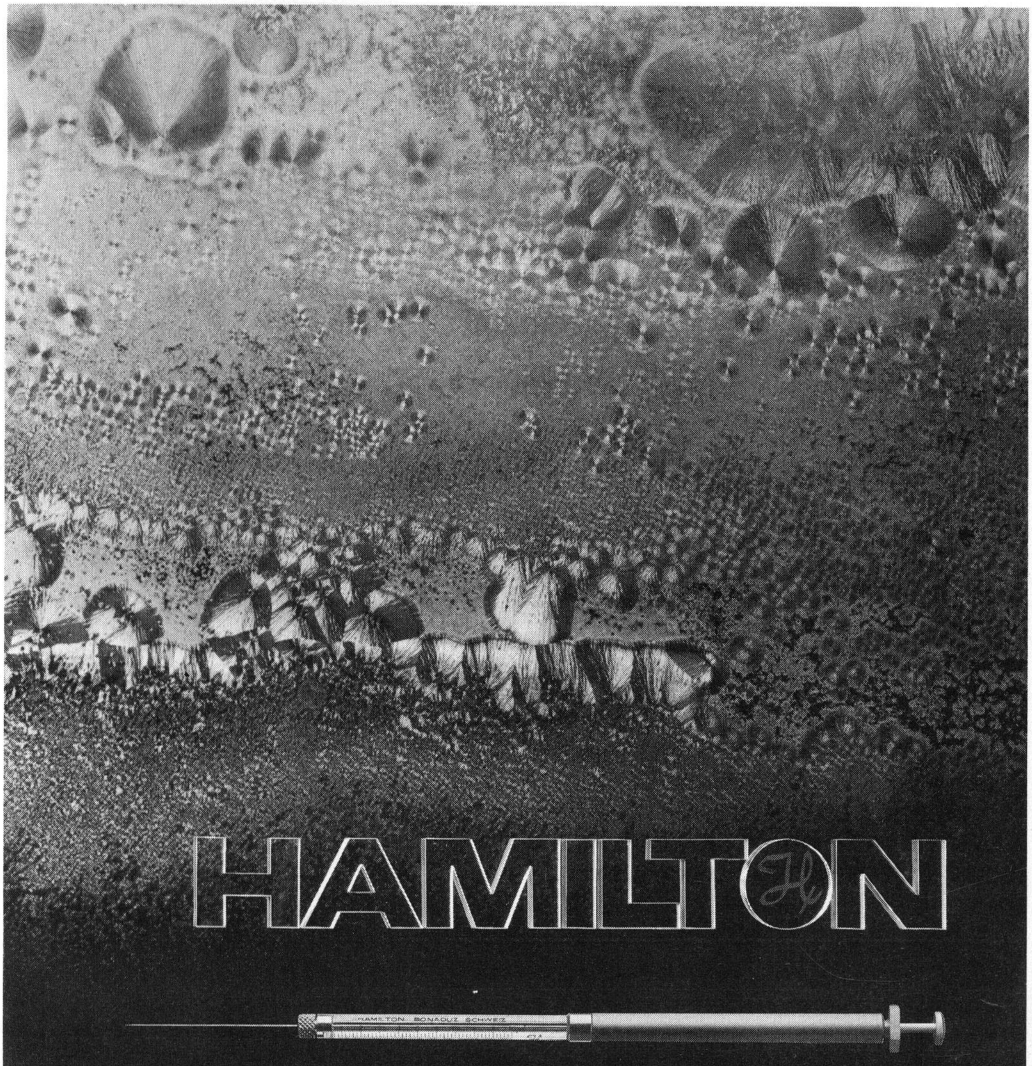
CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin and Science Citation Index.)

(Continued overleaf)

Notes

Letters to the Editor


HAMILTON
HAMILTON BONADUZ SCHWEIZ

Journal of Chromatography

A CHROMATOGRAPHER

is sought for various functions associated with the Journal of Chromatography. The post could take the form of a staff position at Elsevier.

The principal function will be to assist the Editor in the selection of manuscripts for publication in the main section of the Journal.

Applicants should have a good mastery of the English language and a good degree in chemistry, preferably followed by a thesis in chromatography. It is essential that applicants be entirely familiar with the literature of chromatography and have the ability to correspond with authors.

The applicant selected will spend some months each year in the office of the Editor, and the remainder in the Elsevier offices in Amsterdam, where the duties can be varied.

The post can provide a career opportunity for a younger applicant, and can also be of interest to a more senior scientist.

Applications, including a full curriculum vitae and a passport photograph should be sent to:

Dr. M. Lederer
Editor of the Journal of Chromatography
P.O. Box 681
1000 AR AMSTERDAM
The Netherlands

Journal of Chromatography, 247 (1982) 205–210
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,061

REACTIONS OF THERMAL-ENERGY ELECTRONS WITH ORGANIC COMPOUNDS

ANDRÉE CLARET and ALBERT ZLATKIS*
Department of Chemistry, University of Houston, Houston, TX 77004 (U.S.A.)
and
BRIAN S. MIDDLEDITCH
Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX 77004 (U.S.A.)
(Received March 30th, 1982)

SUMMARY

The products resulting from the reaction between electrons of thermal energy and several electron-absorbing organic compounds have been studied. Thermal electrons were generated by maintaining a glow discharge between a stainless-steel electrode and a silver–silver chloride electrode in a nitrogen atmosphere.

The organic compounds investigated fell into two principal classes whose structures contained either simple or conjugated electrophores. The identification of the reaction products by gas chromatography–mass spectrometry showed that simple electrophores, such as nitrobenzene and *o*-dichlorobenzene, react predominantly by a dissociative mechanism while conjugated electrophores, such as benzaldehyde and *trans*-cinnamaldehyde, react predominantly by a non-dissociative mechanism. *m*-Chlorobenzaldehyde, containing both a simple electrophore (chlorine substituent) and a conjugated electrophore (carboxaldehyde moiety), was found to react primarily by a non-dissociative mechanism. *trans*-Cinnamaldehyde, even though possessing a more extensive system of conjugation than benzaldehyde, was found to be less reactive than the latter. Reaction mechanisms consistent with possible pathways of the major products of representative compounds studied are also presented.

INTRODUCTION

In order to provide an adequate theory for the operation of the electron-capture detector (ECD) and to predict its sensitivity to different compounds, the principal problem has always been a lack of knowledge concerning the products formed in the detector by electron attachment. Such products are expected to include neutral molecules, radicals and positive and negative ions.

Several methods have been described that attempt to identify these products. One of them uses a column interposed between two electron-capture detectors for the separation and detection of these products[1,2]. However, only limited information is obtained and failure of the second detector to register products is no proof that they

0021-9673/82/0000–0000/$02.75

do not exist in the first; they may have been totally consumed or they may not have been able to pass through the interposed column. An alternative possibility is that the products with weak electron-capturing properties will not be seen by the second detector.

Another technique for studies of this type is plasma chromatography[3]. Using nitrogen as a carrier gas, the plasma chromatograph creates the negative reactant species of low-energy electrons to react with the gas chromatographic peak component and form characteristic product ions.

In 1970, Durbin *et al.*[4] developed a procedure first introduced by Lovelock in 1962[5]. They studied the products formed by exposing certain compounds to electrons of near-thermal energy generated in a glow discharge. The products formed were identified by gas chromatography–mass spectrometry.

This study is a continuation of these investigations. The identification of the reaction products could help in the understanding of the response of the ECD to different classes of compounds and also, in consequence, aid in the selection of electrophores for the development of new derivatizing agents.

EXPERIMENTAL

A schematic diagram of the apparatus used in this work is shown in Fig. 1.

Nitrogen of ultra-high-purity grade (Iweco, Houston, TX, U.S.A.) was used as the carrier gas and passed through a cartridge packed with molecular sieve, charcoal and calcium sulfate. All of the tubing in the system was made of PTFE to minimize chemical contamination. The flow was controlled by a micro-metering valve to deliver a total flow of 50–60 ml min^{-1}.

The electrical circuit consisted of a high-voltage d.c. voltage supply (Model 230-2P, Sorenson, South Norwalk, CT, U.S.A.) connected in series, first to the discharge cell and then to a microammeter (Model 177 microvolt DMM, Keithly Instruments, Cleveland, OH, U.S.A.) as shown in Fig. 1.

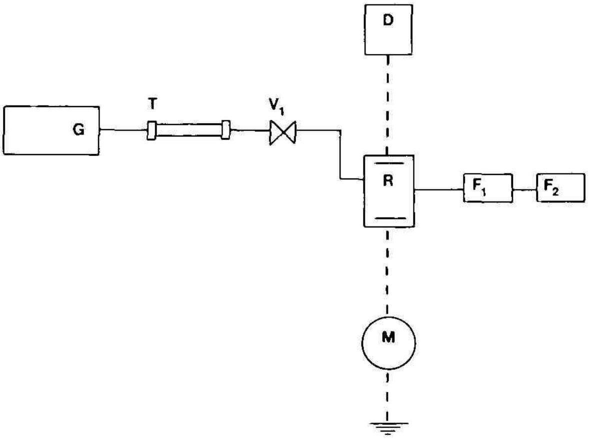


Fig. 1. Diagram of experimental apparatus. ———, Gas circuit; ---, electrical circuit. R = Thermal-electron generator and reaction vessel; D = high-voltage d.c. source; G = gas tank; T = drying tube; V_1 = micrometering valve; F_1 = ball-type flow meter; F_2 = bubble-type flow meter; M = μA meter.

A diagram of the discharge cell is shown in Fig. 2. The system of the electrodes designed yields currents up to 250 μA. The flow discharge is maintained by application of a negative potential of 5–15 kV to the stainless-steel electrode in the gas atmosphere; the inner glass tube allows limited diffusion of vapor molecules from the test substance to the region of ionization around the stainless-steel electrode. It is very important that the surface of this electrode remains perfectly smooth and does not present any sharp edges where the electric field is enhanced, or it will induce arcing. The silver–silver chloride in 1.0 *M* cesium chloride served as a well defined electrode which avoided the possibility of anodic reactions.

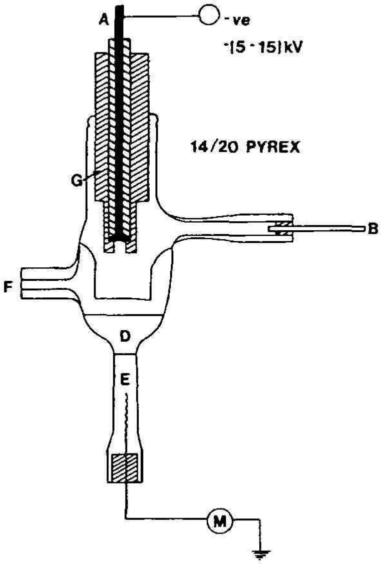


Fig. 2. Generator of free thermal-energy electrons. A = Stainless-steel rod; B = gas inlet; D = test material; E = 1.0 *M* cesium chloride solution containing a silver–silver chloride electrode; F = gas outlet; G = PTFE jacket; M = 0–300 μA meter.

After the reaction had been allowed to proceed for 4 h, the products remaining in the organic layer were analyzed by gas chromatography–mass spectrometry. A Finnigan 1020/OWA instrument was equipped with a 30 m × 0.32 mm I.D. DB-5 bonded-phase fused-silica capillary column (J & W Scientific) programmed from 90 to 300°C at 4°C min^{-1}. The outlet of this column was connected directly into the ion source of the mass spectrometer.

RESULTS AND DISCUSSION

Simple electrophores

Nitrobenzene. Although nitrobenzene has a high relative electron-capture coefficient of 390 (ref. 6), only 15.5% of the starting material reacted. The quantitative distribution of the major products is given in Table I.

The major products formed in this reaction indicate that dissociative capture is the process taking place. When attachment of an electron occurs, a nitro anion

TABLE I

MAJOR REACTION PRODUCTS WITH NITROBENZENE

Molecular weight	*Compound*	*Yield (%)*
94	Phenol	2.5
139	2-Nitrophenol	4.2
139	3-Nitrophenol	3.2
139	4-Nitrophenol	1.7
168	Mixture of 1,3-dinitrobenzene and 1,4-dinitrobenzene	1.9

dissociates from the nitrobenzene molecule, leaving a phenyl radical:

$$C_6H_5NO_2 + e^- \rightarrow C_6H_5^{\cdot} + NO_2^-$$

Possible chain-propagating steps include the following:

(i) reaction of the phenyl radical with water:

$C_6H_5^{\cdot} + H_2O \rightarrow C_6H_5OH + H^{\cdot}$

(ii) formation of the nitrophenyl radical:

$C_6H_5^{\cdot} + C_6H_5NO_2 \rightarrow {}^{\cdot}C_6H_4NO_2 + C_6H_6$

(iii) reaction of the nitrophenyl radical with water:

${}^{\cdot}C_6H_4NO_2 + H_2O \rightarrow HOC_6H_4NO_2 + H^{\cdot}$

(iv) reaction of the nitrophenyl radical with nitrobenzene:

${}^{\cdot}C_6H_4NO_2 + C_6H_5NO_2 \rightarrow O_2NC_6H_4NO_2 + C_6H_5^{\cdot}$

The low overall yield of the reaction could be explained by the fact that the initial step could be reversible.

o-Dichlorobenzene. Fifty-five percent of the starting material reacted. The quantitative distribution of the major products is given in Table II. It appears that *o*-dichlorobenzene reacts via a dissociative mechanism. Possible propagating steps include the following:

$$ClC_6H_4Cl + e^- \rightarrow {}^{\cdot}C_6H_4Cl + Cl^-$$
$${}^{\cdot}C_6H_4Cl + H_2O \rightarrow HOC_6H_4Cl + H^{\cdot}$$
$${}^{\cdot}C_6H_4Cl + HOC_6H_4Cl \rightarrow C_6H_5Cl + {}^{\cdot}C_6H_3ClOH$$
$${}^{\cdot}C_6H_3ClOH + C_6H_4Cl_2 \rightarrow C_6H_3Cl_2OH + {}^{\cdot}C_6H_4Cl$$
$${}^{\cdot}C_6H_4Cl + C_6H_4Cl_2 \rightarrow C_6H_5Cl + {}^{\cdot}C_6H_3Cl_2$$

TABLE II

MAJOR REACTION PRODUCTS WITH *o*-DICHLOROBENZENE

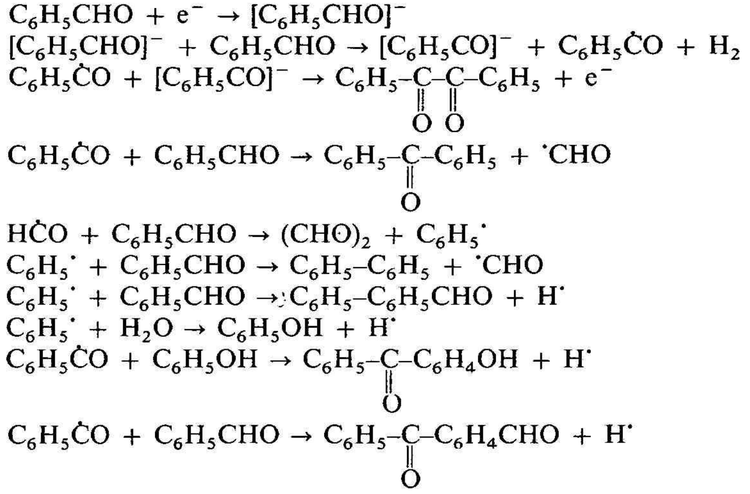
Molecular weight	*Compound*	*Yield (%)*
112	Chlorobenzene	10.6
180	Trichlorobenzene	21.6
162	2,4-Dichlorophenol	0.9
162	2,6-Dichlorophenol	8.8
162	2,3-Dichlorophenol	0.8
162	3,5-Dichlorophenol	5.0
128	3-Chlorophenol	1.5
222 222	Dichloro-1,1′-biphenyl (two isomers)	2.1
256 256	Trichloro-1,1′-biphenyl (two isomers)	2.6

$^{\bullet}C_6H_3Cl_2 + C_6H_4Cl_2 \rightarrow C_6H_3Cl_3 + {}^{\bullet}C_6H_4Cl$
$^{\bullet}C_6H_4Cl + C_6H_4Cl_2 \rightarrow C_6H_4Cl\text{–}C_6H_4Cl + Cl^{\bullet}$
$^{\bullet}C_6H_4Cl + C_6H_3Cl_3 \rightarrow C_6H_4Cl\text{–}C_6H_3Cl_2 + Cl^{\bullet}$

Comparing the overall yield for the reaction with *o*-dichlorobenzene with the one obtained by Durbin *et al.*[4] with chlorobenzene, it appears that the presence of a second chlorine atom on the benzene ring enhances the reactivity towards electron capture.

Conjugate electrophores

Benzaldehyde. Forty percent of the starting material reacted. The quantitative distribution of the major products is given in Table III. It shows that non-dissociative electron capture is the predominant mechanism, as indicated by the following:



$$C_6H_5CHO + e^- \rightarrow [C_6H_5CHO]^-$$
$$[C_6H_5CHO]^- + C_6H_5CHO \rightarrow [C_6H_5CO]^- + C_6H_5\dot{C}O + H_2$$
$$C_6H_5\dot{C}O + [C_6H_5CO]^- \rightarrow C_6H_5\text{–}\underset{\substack{\|\\O}}{C}\text{–}\underset{\substack{\|\\O}}{C}\text{–}C_6H_5 + e^-$$
$$C_6H_5\dot{C}O + C_6H_5CHO \rightarrow C_6H_5\text{–}\underset{\substack{\|\\O}}{C}\text{–}C_6H_5 + {}^{\bullet}CHO$$
$$H\dot{C}O + C_6H_5CHO \rightarrow (CHO)_2 + C_6H_5^{\bullet}$$
$$C_6H_5^{\bullet} + C_6H_5CHO \rightarrow C_6H_5\text{–}C_6H_5 + {}^{\bullet}CHO$$
$$C_6H_5^{\bullet} + C_6H_5CHO \rightarrow C_6H_5\text{–}C_6H_5CHO + H^{\bullet}$$
$$C_6H_5^{\bullet} + H_2O \rightarrow C_6H_5OH + H^{\bullet}$$
$$C_6H_5\dot{C}O + C_6H_5OH \rightarrow C_6H_5\text{–}\underset{\substack{\|\\O}}{C}\text{–}C_6H_4OH + H^{\bullet}$$
$$C_6H_5\dot{C}O + C_6H_5CHO \rightarrow C_6H_5\text{–}\underset{\substack{\|\\O}}{C}\text{–}C_6H_4CHO + H^{\bullet}$$

m-Chlorobenzaldehyde. After studying, successively, simple electrophores and conjugate electrophores, and noticing that they reacted via dissociative and non-dissociative mechanisms, respectively, it was of interest to study how a compound containing both would react.

In the reaction with *m*-chlorobenzaldehyde, 22% of the starting material reacted. The quantitative distribution of the major products is given in Table IV.

The overall reactivity of *m*-chlorobenzaldehyde towards electron capture is lower than that of benzaldehyde. From the identified products, it appears that non-

TABLE III

MAJOR REACTIONS PRODUCTS WITH BENZALDEHYDE

Molecular weight	*Compound*	*Yield (%)*
154	Biphenyl	4.2
182	Benzophenone	8.0
182	Biphenyl carboxaldehyde	4.7
182	(two isomers)	
198	Hydroxybenzophenone	3.4
210	Benzil	9.0
210	Benzophenone carboxaldehyde	5.1

TABLE IV

MAJOR REACTION PRODUCTS WITH *m*-CHLOROBENZALDEHYDE

Molecular weight	*Compound*	*Yield (%)*
128	Chlorophenol	5.1
250	4,4′-Dichlorobenzophenone	1.5
278	Dichlorobenzil	1.3
264	Chlorodeoxybenzoin	1.8

dissociative electron capture is the predominant mechanism (formation of dichlorobenzophenone and dichlorobenzil).

trans-Cinnamaldehyde. The overall yield of the reaction with *trans*-cinnamaldehyde was 23%, and the quantitative distribution of the major products is given in Table V.

TABLE V

MAJOR REACTION PRODUCTS WITH *trans*-CINNAMALDEHYDE

Molecular weight	*Compound*	*Yield (%)*
134	Phenylpropanal	2.9
162	Phenylpropanoic acid	2.3
248	4-Benzyl-2-carboxy-1-hydronaphthalene	6.8
266	1,6-Diphenyl-3,4-hexanedione and another isomer	1.9

As the major product formed is a hydrogenated dimer of cinnamaldehyde, the predominate mechanism is non-dissociative. Two hydrogenation reactions occur: formation of phenylpropanal by hydrogenation of cinnamaldehyde and formation of phenylpropanoic acid by hydrogenation of phenylpropenoic acid (impurity in the starting material).

The overall yield of the reaction is lower than that of benzaldehyde. This indicates that, even though there is a more extensive conjugated system in the reaction with *trans*-cinnamaldehyde, more conjugation does not imply higher reactivity towards electron capture.

CONCLUSION

The glow discharge is a simple, straightforward method of studying the reactions of thermal-energy electrons with organic compounds. The main advantage over methods previously described[2] is that the products of the reaction are produced in sufficient yield to be recovered and characterized. Thus, the different mechanisms involved were determined.

REFERENCES

1 S. Kapila and W. A. Aue, *J. Chromatogr.*, 108 (1975) 13.
2 S. Kapila and W. A. Aue, *J. Chromatogr.*, 148 (1978) 343.
3 F. W. Karasek, O. S. Tatone and D. W. Denney, *J. Chromatogr.*, 87 (1973) 137.
4 D. E. Durbin, W. E. Wentworth and A. Zlatkis, *J. Amer. Chem. Soc.*, 92 (1970) 5131.
5 J. E. Lovelock, *Nature (London)*, 195 (1962) 488.
6 J. E. Lovelock, *Nature (London)*, 189 (1961) 729.

Journal of Chromatography, 247 (1982) 211–219
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,063

EVALUATION OF MULTI-STAGE GAS CHROMATOGRAPHY IN QUANTITATIVE CHEMICAL ANALYSIS

JOSEF F. K. HUBER*, ERNST KENNDLER and WOLFGANG NYIRY
Institute of Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna (Austria)
and
M. OREANS
Siemens AG, Bereich Prozess- und Messtechnik, D-7500 Karlsruhe (G.F.R.)

(Received April 20th, 1982)

SUMMARY

Two-stage gas chromatography has proved to be a powerful means for the isolation of trace components from complex mixtures. The application of this technique in quantitative analysis has not yet been evaluated and is considered in this paper. The influence of the size and position of the transferred fraction on the quality of the analytical data was investigated. A compromise between low peak interference and high transfer efficiency has to be found. It is shown that the same ultimate precision and accuracy can be obtained with column switching as in single-column operation provided that a high-performance switching device and appropriate fractionation are used.

INTRODUCTION

In order to separate a complex mixture by chromatography, the first requirement is to achieve a separation performance that is at least as large as the number of components to be separated by a given minimum resolution. In practice, the separation performance should be significantly larger than the number of components of the sample. The separation performance, n_{Rt}, is defined as the maximum number of components, n, that can be separated with a given resolution, R, in a given time, t. The separation performance depends on the average theoretical plate number, $\bar{N}$, of the column and is given by the following equation[1,2]:

$$n_{Rt} = \frac{\log (t_{Rn}/t_{R0})}{\log (1 + R/\sqrt{\bar{N}})} = \frac{\log (1 + \kappa_n)}{\log (1 + R/\sqrt{\bar{N}})} \qquad (1)$$

where t_{Ri} = retention time of component, i; $i = 0,1,\ldots, n$.

The subscript zero indicates a non-retarded component with capacity factor $\kappa_i = 0$; the subscript n, indicates the last eluting component with the highest capacity factor, κ_n = maximum. From eqn. 1, it can be seen that the separation performance depends on the retention range, $t_{Rn}/t_{R0} = 1 + \kappa_n$, as well as on the average theoretical plate number of the column, $\bar{N}$. The individual theoretical plate number, N_i, of the components, i, depends not only on the chromatographic column but also on the sample component, as the capacity factor, κ_i, and the diffusion coefficients, $D_{\mathrm{m}i}$ and D_{si}, in the mobile phase and in the stationary phase, respectively, occur in the equation of the theoretical plate number[3,4].

In order to obtain a value for the dispersion characteristic of the column that is dependent only on the system and independent of the sample, it is necessary to estimate the average of the theoretical plate number, $\bar{N}$, from the measured individual values, N_i, of a number of components. The best average value, $\bar{N}$, is obtained by linear regression of the peak variances and the squares of the retention times according to

$$\sigma_{ti}^2 = \bar{N}\, t_{Ri}^2 \tag{2}$$

where σ_{ti}^2 is the variance of the elution peak of component i. The slope, $\bar{N}$, of the linear regression line of σ_{ti}^2 *versus* t_{Ri}^2 depends only slightly on the choice of the test compounds if the diffusion coefficients and therefore the individual values, N_i, of these compounds do not vary too much.

If the first requirement to make the separation performance for a given time interval sufficiently large compared with the number of components to be separated, $n_{Rt} \gg n_{\mathrm{S}}$, is fulfilled, then the second requirement is to utilize fully this separation performance. For this purpose the elution sequence of the components has to be adjusted within the given time interval via the selectivity of the chromatographic phase system. The aim is to arrange the components in such an order that all of them are separated at least according to the required resolution. It is obvious that this target can be reached more easily the larger the separation performance is, compared with the number of components to be separated, $n_{Rt} \gg n_{\mathrm{S}}$.

Often it is not possible, however, to find a phase system that is sufficiently selective for all components of the mixture and the required minimum resolution cannot be obtained for all components. In such instances the required degree of separation can be achieved by gradual adjustment of the column selectivity. The separation is carried out in several stages on different columns. This multi-stage chromatography can be executed on-line by column switching where the effluent of a column of a given stage is divided into fractions which are transferred to the detector or to the columns of the next stage by means of a switching device. If columns with different retention characteristics are used in the individual stages of a separation path the multi-stage technique is called multi-dimensional chromatography.

Multi-stage chromatography can also be used with advantage if columns with the same retention characteristics are used in each stage. In this technique, a given component is enriched relative to interfering constituents of the sample by fractionation. An effluent fraction containing the analyte component and as little as possible interfering components is transferred from the first to the second column on which the separation of the fraction can be completed owing to the more favourable peak size ratio. This relative enrichment has the result that the analytical information parameters (retention time, peak area) can be determined with a higher accuracy than in a single-stage separation giving the same resolution. Correspondingly, a lower resolution is required to obtain a given accuracy.

Multi-stage gas chromatography was applied first as a relative enrichment technique (heart cutting)[5] and later as a multi-dimensional method[6–10]. The performance of multi-stage gas chromatography in quantitative analysis has never been evaluated. It was our intention to investigate the quality of the analytical data in multi-stage gas chromatography compared with single-column operation using advanced instrumentation.

EXPERIMENTAL

Chemicals

All chemicals used were of analytical-reagent grade from E. Merck (Darmstadt, G.F.R.), except decahydronaphthalenes, which were obtained from EGA-Chemie (Steinheim, G.F.R.). *Cis*- and *trans*-decahydronaphthalene diluted with toluene to a content of 10% (v/v) with addition of *n*-tridecane as internal standard and a commercially available lavender oil diluted to 10% (v/v) with 2,2,4-trimethylpentane were used as test samples. The carrier gas used in gas chromatography was nitrogen of 99.995% (v/v) purity (Messer-Griesheim, Düsseldorf, G.F.R.).

The polydimethylsiloxane OV-101 (E. Merck) and the aromatic polyether Marlophen (Hüls, Marl, G.F.R.) were used as stationary phases.

Apparatus

A gas chromatograph equipped with two flame-ionization detectors and a switching device was used (Model L402, Siemens, Karlsruhe, G.F.R.). The switching device is described later. Two capillary columns were used in the switching operation. The chromatograms were recorded by means of a two-channel potentiometric line recorder (Siemens, Kompensograph X–T). Retention-times, peak-areas and switching intervals were measured with the aid of a computing integrator (Model Autolab, System I, Spectra Physics, Santa Clara, CA, U.S.A.).

The following chromatographic conditions were used for the determination of the precision and accuracy of the two-stage switching procedure depending on the size and position of the fraction: two open-tubular columns, coated with OV-101, lengths 18 and 23 m, I.D. 0.3 mm; column temperature, 95 and 130°C, respectively; temperature of injector and detector, 200°C; splitting ratio, 1:100.

The average efficiencies of the columns were measured as discussed in the Introduction and gave values of $\bar{N}$ = 55,000 for the 18-m column and $\bar{N}$ = 125,000 for the two columns (18 + 23 m) in series.

In the application of the relative enrichment procedure to the analysis of trace components in lavender oil, the gas chromatographic conditions were as follows: two open-tubular columns, both coated with Marlophen, lengths 18 and 23 m, I.D. 0.3 mm; $\bar{N}$ = 57,000 for the 18-m column, $\bar{N}$ = 98,000 for the two columns (18 + 23 m) in series; splitting ratio, 1:100; temperature programme, linear gradient from 60 to 160°C at the rate of 5°C min^{-1}; injector and detector temperature, 220°C.

The carrier gas flow-rate, measured at the outlet of the second column at ambient temperature and pressure, was 2 ml min^{-1} in both instances, with p_0 = 1.8 bar and p_B = 1.6 bar.

The switching device was an improved and optimized version of the valveless design as proposed by Deans[5], and is shown schematically in Fig. 1. The interface is described in detail elsewhere[11]. It consists of a double T-piece with tube connections for the adjustment of variable pressures and a platinum capillary (2.4 cm × 0.1 mm I.D.) for the connection of the two capillary columns.

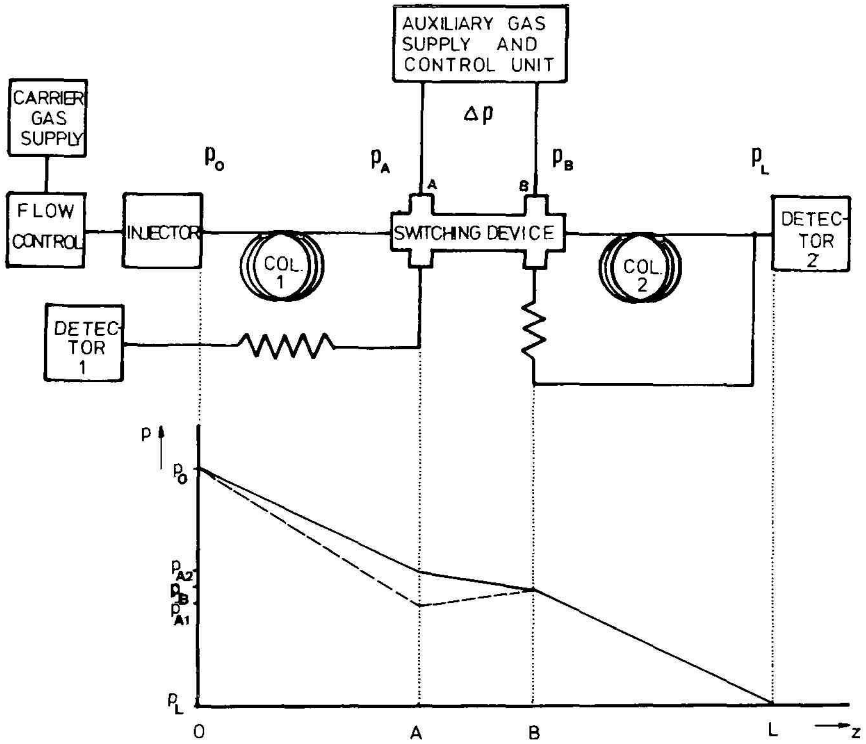


Fig. 1. Schematic diagram of a gas chromatograph for column switching. p = pressure; z = length; p_0 = pressure at the inlet of the first column ($z = 0$); p_A, p_B = pressures at points A and B, respectively, of the switching device; p_L = pressure at the outlet of the second column ($z = L$). The pressure difference, Δp, is adjusted with the aid of the auxiliary gas supply unit and equals $p_{A1} - p_B$ for single-stage operation and $p_{A2} - p_B$ for two-stage operation. For details, see Experimental.

The direction of flow in the connecting tube between the two columns is controlled by variable pressure levels. The pressure, p_0, at the inlet of the first column is kept constant and adjusted by a pressure controller. The pressure, p_B, at the outlet of the interface is identical with the inlet pressure of the second column. It is kept constant and is also adjusted with the aid of a pressure controller. In order to direct the flow from the first column either to the first detector or to the second column and the second detector the pressure, p_A, at the inlet of the interface is varied between two levels, p_{A1} and p_{A2}, by means of a pressure controller.

In the single column mode, where the effluent from the first column is directed to the first detector, a pressure drop, Δp, is adjusted with the aid of the pressure controller unit, which sets point A of the interface to a pressure, p_{A1}, a few millibars lower than the pressure at point B. The pressure drop Δp is measured by means of a digital pressure meter. Owing to the pressure difference, the effluent from column 1 is hindered from entering column 2 and is forced to flow to the monitoring detector, over a constriction capillary.

In order to cut out a fraction of the effluent from column 1, which is recorded on detector 1, the pressure at point A is rapidly increased to a value, p_{A2}, allowing flow to the second column. After transferring the fraction of interest into the second column, the pressure at point A is set back to the lower value, interrupting the flow of the effluent to the second column. As the flow of carrier gas in none of the columns is interrupted, the elution proceeds continuously.

The great advantage of the switching device is the negligible dead volume, the very high speed of switching and the absence of mechanical valves in the flow path of the sample.

RESULTS AND DISCUSSION

The information parameters in quantitative chemical analysis by means of chromatography are peak area or peak height. The quality of these data in two-stage gas chromatography applying relative analyte enrichment by column switching was investigated. This technique was applied to the determination of minor components in a matrix of interfering major components. A system of two columns of the same type connected by a switching valve was used, the effluent from each column being led to a separate detector. The sample was injected into the first column where the analytes were partially separated from interfering matrix components. The fraction containing the analytes was transferred to the second column, where the separation was completed. A complete separation can only be achieved by this two-stage operation and not by a single column with the total length of the two columns. The superior separation effect by the two-stage operation is caused by the fact that in the second stage the separation is continued with a significantly improved ratio of the peak height of the analyte to those of the overlapping matrix components. The resolution in both modes of operation remains the same but the peak-height ratio is significantly improved in two-stage operation, leading to a better degree of separation of the analytes. In general, several analytes can be isolated in a single run. For the determination of many components several runs are required.

It can be expected that both the accuracy and the precision of the analytical data in column switching will depend on three factors: the technical characteristics of the switching device, the size of the fraction and the position of the fraction. The effects of these three factors on the precision and accuracy of peak area and peak height are discussed below for both isolated peaks and interfering peaks.

Fractionation of isolated peaks

The influence of the fraction size on the analytical results in two-stage gas chromatography was first investigated for the transfer of a single, isolated peak in order to define the optimum switching conditions and to test the performance of the switching device. The peak maximum of component 1, eluting first from the first column, was used as a marker for the measurement of the switching time. The second eluting component 2 was fractionated by means of a switching valve. The fraction was transferred from the first to the second column and measured in its effluent by the second detector. Quantitation of this compound was carried out based on the peak-

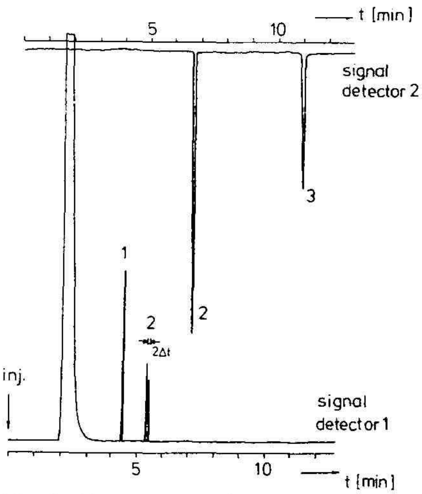


Fig. 2. Chromatogram from a two-stage gas chromatograph with two-channel detection. Compound 1 (*trans*-decahydronaphthalene) was used as a marker substance for the measurement of the switching time, $2\Delta t$, for the transfer of fractions of compound 2 (*cis*-decahydronaphthalene) from column 1 to column 2. Compound 3 (tridecane), used as reference substance in the quantitation of compound 2, was totally transferred to column 2. t = time.

area measurement obtained from the second detector. The third eluting component 3 was transferred to the second column without fractionation and was used as a reference in the quantitation of the second component. The precision and accuracy of the area of the peaks occurring in the chromatogram from the second detector were determined relative to the area of the totally transferred third peak. An example of such a two-channel chromatogram is shown in Fig. 2, and the results for precision and accuracy are given in Table I and are shown graphically in Fig. 3.

TABLE I

DATA ON THE PRECISION AND ACCURACY OF COLUMN SWITCHING IN GAS CHROMATOGRAPHY FOR ISOLATED PEAKS

Switching was carried out for half-intervals of $\Delta t = n\sigma_t$ symmetrical to the expected position of the peak maximum. σ_t = peak standard deviation in time units; n = 1,2,3...

Peak standard deviation σ_t (sec)	*Total switching interval $2\Delta t$ (sec)*	*Relative switching half-interval $n = \Delta t/\sigma_t$*	*No. of measurements*	*Relative statistical error, s (%)*	*Yield (% of total peak area)*
1.1	2.2	1	9	2.50	48.7
	4.4	2	8	1.78	77.7
	6.6	3	6	0.94	93.6
	8.8	4	5	0.63	98.6
	11.0	5	6	0.42	100.2
	13.2	6	8	0.33	100.1
2.0	4.0	1	6	1.10	59.5
	8.0	2	5	0.75	92.4
	12.0	3	6	0.42	99.6
	16.0	4	5	0.28	99.8
	20.0	5	4	0.21	99.8

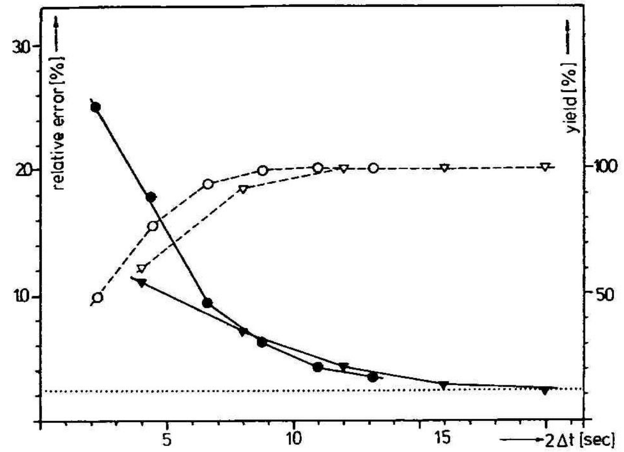


Fig. 3. Precision and accuracy of column switching in gas chromatography for an isolated peak. Measurements were carried out for peaks with standard deviations of 1.1 sec (●, ○) and 2.0 sec (▼, ▽). $2\Delta t$ = total switching time, with half-intervals of Δt sec symmetrical to peak maximum. Dotted line: limiting value of the relative error measured in single-stage operation. Closed symbols, relative error; open symbols, yield.

It can be seen that for a peak standard deviation of 2 sec the systematic error due to the switching operation reduces to below −0.5% if the switched fraction exceeds six times the peak standard deviation and has a symmetrical position to the centre of the peak. With a fraction size of ten times the peak standard deviation the transfer yield is 99.8%. For a peak standard deviation of 1.1 sec the systematic error is −1.4% for a fraction size corresponding to eight times the peak standard deviation.

It can also be seen in Fig. 3 that the statistical error decreases together with the systematic error and approaches a limiting value of 0.24% for a sufficiently large fraction size. The same precision was also found for single stage operation without switching.

The results indicate that the switching device has no effect on the precision and accuracy of the data in quantitative analyses, assuming that the fraction size is large enough. We recognize, however, that the precision depends primarily on the absolute fraction size in seconds. It seems that the minimum time interval for column switching without loss in precision is about 1 sec.

Fractionation of overlapped peaks

The choice of the size and position of a fraction becomes extremely important if an analyte is overlapped by matrix components. In such a case a compromise has to be found for the fractionation step after the first column. On the one hand, the analyte should be transferred to the second column by means of the switching device as completely as possible in order to achieve a high precision; on the other hand, the amount of overlapping matrix components should be reduced as much as possible in order to achieve a high accuracy. The first aim requires a large and the second a small fraction size. This situation requires the optimization of the fraction size depending on the sample.

Such an optimization will be demonstrated in the determination of a minor constituent in lavender oil. Even with high-efficiency open-tubular column gas chromatography this component appears in the chromatogram as a shoulder on a large peak. It can be assumed that the minor component can be isolated from the interfering large component by a two-stage operation applying relative enrichment. In order to combine high accuracy and high precision in the determination of this component, the optimum fraction size has to be used in the transfer from the first to the second separation stage.

In Fig. 4 the chromatograms obtained with different fraction sizes are shown, assuming a constant end-point of the fraction. It can be seen that the overlapping by the large peak can be reduced to an insignificant effect and practically complete isolation of the analyte peak can be achieved. It can also be seen that, as expected, the peak height of the analyte peak increases with decreasing fraction size, approaching a maximum value. Below the fraction size corresponding to the total amount of analyte the peak height starts to decrease, as only part of the analyte is transferred and detected. The effect of the overlapping is already neglegible for the fraction size corresponding to Fig. 4C, as can be concluded from the comparison of the peak heights in Fig. 4C and D.

The precision of the system and the relative independence of the results of slight variations in the position and size of fraction are shown in Fig. 5. We recognize the very good reproducibility of the peak height for the analyte although the patterns

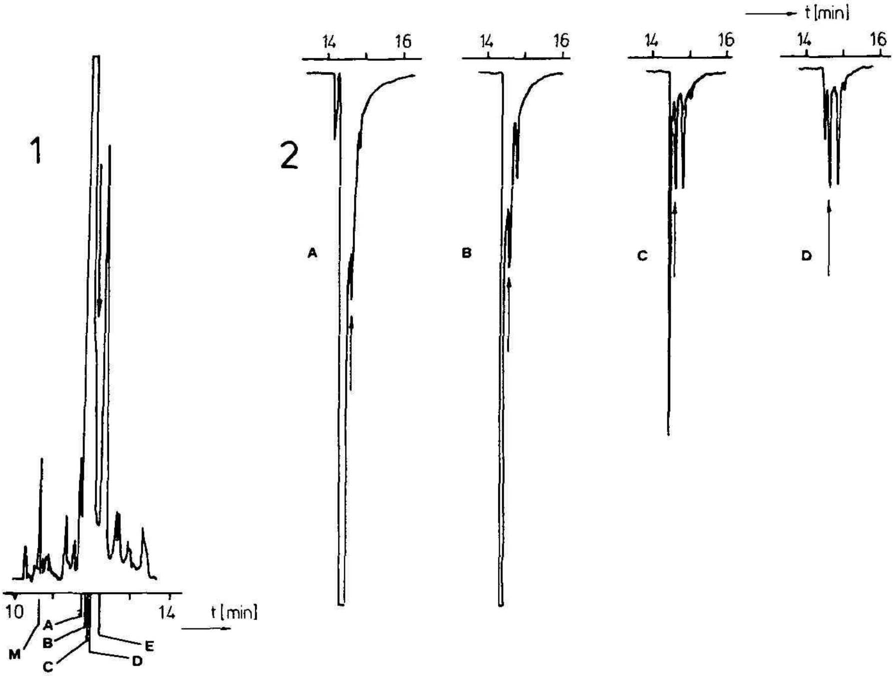


Fig. 4. Influence of the fraction size in column switching on the peak interference in the second stage. A minor component in lavender oil, indicated by arrows; appears as a shoulder in the chromatogram of column 1. The time, E, of the end of the switching interval was held constant at 97.1 sec, measured from the maximum of the marker peak, M. The start, A, B, C and D, of the switching interval was varied: A = 62.1 sec; B = 72.1 sec; C = 73.1 sec; D = 75.1 sec; measured from the peak maximum of M. 1, Part of the chromatogram from column 1, recorded on detector 1; 2, chromatograms of the different fractions, A–E, B–E, C–E and D–E, switched from column 1 to column 2 and recorded on detector 2. t = time.

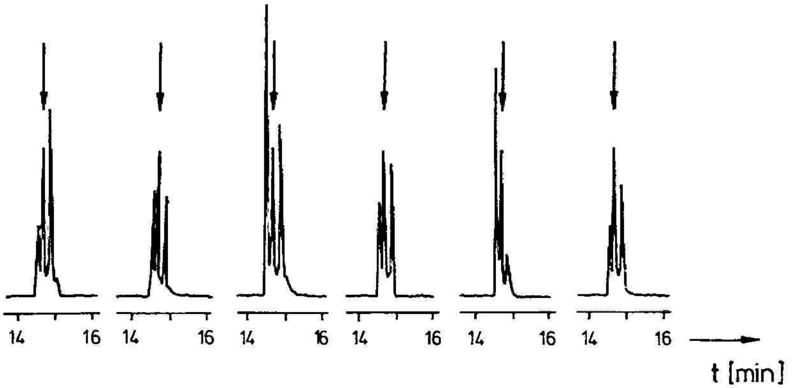


Fig. 5. Reproducibility of column switching in gas chromatography with open-tubular columns. The minor component, as shown in Fig. 4, was switched to column 2 with an average switching time interval of 23 sec. The position and size, respectively, of the switching interval were varied in the range of 2 sec. t = time.

of the chromatograms vary significantly owing to variations in the position of the fractions.

The evaluation of a large number of data leads to the conclusion that high precision and high accuracy can be obtained by choosing the optimum fraction size and position. An example of the choice of the optimum fraction is shown in Fig. 4, where it can be seen that in this instance the optimum switching interval is 23 sec, giving a precision of 1.2 % and an accuracy of 99.8 % for the relative peak height. The absolute values of precision and accuracy are less favourable owing to the sampling error.

REFERENCES

1 J. F. K. Huber and H. C. Smit, *Z. Anal. Chem.*, 245 (1969) 84.
2 J. F. K. Huber, E. Kenndler and G. Reich, *J. Chromatogr.*, 172 (1979) 15.
3 J. J. van Deemter, F. J. Zuiderweg and A. Klinkenberg, *Chem. Eng. Sci.*, 5 (1956) 271.
4 M. Golay, in D. H. Desty (Editor), *Gas Chromatography 1958*, Butterworths, London, 1958, p. 36.
5 D. R. Deans, *Chromatographia*, 1 (1968) 18.
6 J. D. Rynbrandt, D. F. Ring and B. S. Rabinovitch, *J. Gas Chromatogr.*, 6 (1968) 531.
7 H. Boer, in S. G. Perry (Editor), *Gas Chromatography*, Applied Science, Barking, 1973, p. 109.
8 G. Schomburg, H. Husmann and F. Weeke, *J. Chromatogr.*, 112 (1975) 205.
9 J. F. K. Huber, E. Kenndler and H. Markens, *J. Chromatogr.*, 167 (1978) 291.
10 W. Bertsch, in W. Bertsch, W. G. Jennings and R. E. Kaiser (Editors), *Recent Advances in Capillary Gas Chromatography*, Hüthig, Heidelberg, 1981, p. 3.
11 F. Müller and M. Oreans, *Analytical Application Note No. 282*, Siemens, Karlsruhe, 1981.

Journal of Chromatography, 247 (1982) 221–229
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,059

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SACCHARIN, CAFFEINE AND BENZOIC ACID USING NON-LINEAR PROGRAMMING

J. W. WEYLAND*, H. ROLINK and D. A. DOORNBOS
Research Group Optimization, Laboratory for Pharmaceutical and Analytical Chemistry, State University, A. Deusinglaan 2, 9713 AW Groningen (The Netherlands)

(First received December 28th, 1981; revised manuscript received May 24th, 1982)

SUMMARY

A method is described which allows a chromatographic assay to be optimized with respect to the time needed to complete an analysis, while maintaining good resolution. The method introduces an operational research technique called non-linear programming. It was used to optimize the eluent composition for the assay of saccharin, caffeine and benzoic acid and resulted in a significant reduction in the analysis time.

INTRODUCTION

In routine chromatography, analysis time is an important optimization criterion, especially for automated equipment, requiring little or no operator action, as any savings in time will lower the cost per analysis by an equivalent amount. As most published high-performance liquid chromatographic (HPLC) determinations are probably not optimized with respect to either separation quality or time, it may be expected that many of these methods can be improved considerably. This study demonstrates this, using a non-linear programming technique to optimize the time needed to complete the separation of saccharin, caffeine and benzoic acid.

THEORETICAL

Once the column (with a particular stationary phase) has been chosen, the main variable influencing separation is the composition of the mobile phase. This variable also influences the time needed for an analysis, indicating an interaction between the quality of the separation and the analysis time. Retention of a solute depends on its capacity factor, which in turn depends on the "polarity" of the mobile phase.

Polarity has been defined in various ways[1], but can be understood as summarizing the ability of the solvent to interact with the solute. To quantify "polarity" several scales have been proposed, *e.g.*, Snyder's polarity parameter P' (ref. 2), based

0021-9673/82/0000–0000/$02.75

on the Rohrschneider data set, and the Hildebrand solubility parameter[1,3,4]. Both scales hide as much as they reveal, as interactions between solute and solvent are combinations of several different types of interactions: dispersion, orientation, induction and acid–base. In solubility theory, the resultant of these interactions is called the solubility parameter, δ (Snyder[3] described a slightly different scheme, subdividing P' into different intramolecular contributions, x_i).

Schoenmakers[4,5] developed the solubility parameter model to account for the different types of interactions. In their model, each type of interaction is assigned its own solubility parameter. They derived the following equation for the separation factor of two solutes, j and i:

$$\frac{RT}{2v} \cdot \ln \alpha_{j,i} = (\bar{x}_i - \bar{x}_j)\bar{y}$$

where R is the gas constant, T is temperature, v is the molar volume of solutes ($v_i = v_j$ assumed) and $\bar{x}$ and $\bar{y}$ are vectors representing the partial solubility parameters for the solutes and a given mobile–stationary phase system:

$x_1 = \delta_d$, solubility parameter for dispersion — $y_1 = (\delta_{d,m} - \delta_{ind,m}) - (\delta_{d,s} - \delta_{ind,s})$
$x_2 = \delta_0$, solubility parameter for orientation — $y_2 = \delta_{0,m} - \delta_{0,s}$
$x_3 = \delta_{ind}$, solubility parameter for induction — $y_3 = \delta_{d,m} - \delta_{d,s}$
$x_4 = \delta_a$, solubility parameter for acid–base interaction — $y_4 = \delta_{b,m} - \delta_{b,s}$
$x_5 = \delta_b$, solubility parameter for base–acid interaction — $y_5 = \delta_{a,m} - \delta_{a,s}$

where m = mobile phase and s = stationary phase.

Partial differentiation with respect to the different types of interactions yields

$$\frac{RT}{2v} \cdot \frac{1}{\alpha_{j,1}} \cdot \frac{\partial \alpha_{j,i}}{\partial y_n} = x_{n,i} - x_{n,j}$$

where n indicates the type of interaction[5]. This equation shows that the relative change in $\alpha_{j,i}$ resulting from a change in the nth type of interaction is directly proportional to the difference in the nth parameter of the two solutes. This offers the key to exploiting the differences between the solutes: find a mobile phase that maximizes α. In real chromatograms, where there are more peaks to separate, find a mobile phase in which the y-vector is such that it gives an optimum separation for all the peaks. This can be attained more or less by mixing solvents that differ in their "interaction patterns", or stated differently, that lie apart as much as possible in the five-dimensional vector space of the interactions.

Practically stable mixtures in reversed-phase (RP) HPLC can only be made of water, methanol, acetonitrile and tetrahydrofuran. In this study, a mixture of water, methanol and acetonitrile was chosen, largely from cost considerations. It proved adequate, however, for the purpose intended.

EXPERIMENTAL

As the purpose of this study was to optimize analysis time, while maintaining the overall quality of separation, *i.e.*, baseline resolution for all peaks or 99.8% resolution for all peaks, it is natural to constrain the variables to an area in which the desired separation is met, the feasible region, and then find within this area the minimum analysis time.

This indicates the use of an experimental design that allows the mapping of both the optimizing criterion and the resolutions between peaks at the same time, with only one set of experiments. For ternary mixtures a suitable design is the simplex lattice design, as described by Gorman and Hinman[6] or Snee[7]. In chromatography, this experimental design was used by Belinky[8] and Glajch *et al.*[9]. Belinky improved the resolutions between pairs of polynuclear aromatic hydrocarbons at near constant capacity factor, while Glajch *et al.* mapped the resolutions between all pairs of peaks in order to locate a region in the factor space comprised by a (pseudo-)quaternary mobile phase where every resolution meets a predetermined value. Glajch *et al.* did not try to minimize analysis time. Moreover, in their approach this would not be possible, as the water content of the mobile phase (the variable that influences the elution time the most) is not a free variable in their (pseudo-)quaternary system.

A simplex lattice design generally consists of a uniform distribution of points over all possible mixtures. At these points the responses are measured (Fig. 1). Response is then represented by polynomials fitted by least squares to the responses, which serve to map the response surfaces.

Because in mixtures the sum of fractions must add up to unity, it is possible to simplify the polynomials compared with those normally used. A full quadratic can be reduced using the constraint $x_1 + x_2 + x_3 = 1$ to the following relationship:

$$\eta = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

The minimum number of experiments necessary to obtain estimates of the regression parameters is seen to be six, as opposed to nine for a full quadratic polynomial. For higher order equations a similar reduction in the number of experiments is possible. Of course, this reduction is possible because the actual factor space is two-dimensional; the variables are interdependent.

As indicated before, a quadratic simplex lattice design consists of a minimum of six experimental points (Fig. 1). A few extra points should be measured in order to evaluate the goodness of fit of the model.

It is impossible to explore all the accessible factor space in the ternary mixtures used in RP-HPLC (mixtures of water, methanol, tetrahydrofuran and acetonitrile). Experiments with pure water exhibit extremely long elution times, while pure organic solvents will elute most peaks together with the solvent front. The region of interest is thus restricted to a small region of the available factor space.

Depending on the quality and analysis time of the initial chromatogram, a simplex lattice design is located around the original factor point, or a suitable region is located, whereafter the simplex lattice design is set up in this region.

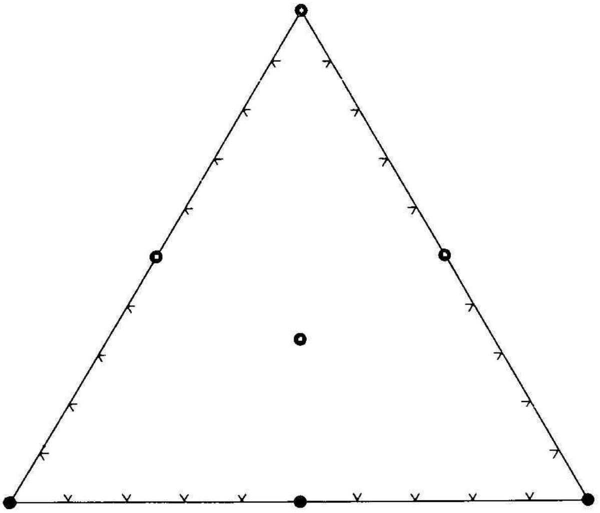


Fig. 1. Quadratic simplex lattice design, central point added.

Separation of sacharine, caffeine and benzoic acid in beverages

For every measured solvent composition the analysis time and the resolutions between all pairs of adjacent peaks were calculated. Analysis time was defined for the purpose of this study as the time it took to elute 99.8 % of the last peak, *i.e.*, time was measured at three times the width at 0.6 height after the top of the last peak. Resolutions were calculated from

$$R_{1,2} = \frac{2(t_2 - t_1)}{(w_1 + w_2)}$$

where w = peak width at the base.

Chemicals

For the mobile phase mixtures of deionized water and analytical-reagent grade methanol and acetonitrile (Merck, Darmstadt, G.F.R.) (degassed under vacuum, using an ultrasonic bath) were used, with 1 % of analytical-reagent grade acetic acid (Merck) added in order to convert the acids to their non-ionized forms. Standard solutions of saccharin (1.0 mg/ml), caffeine (1.0 mg/ml) and sodium benzoate (1.0 mg/ml) and a mixture containing 100 mg of these compounds in 100 ml were prepared using methanol as solvent. Standards were of European Pharmacopoeia quality and were used as received.

Instrumentation

The HPLC apparatus was constructed from a Milton-Roy pump and a Chromatronics 220 dual-channel UV absorbance detector (fixed wavelength, 254 nm). The column used was 25 cm × 4.6 mm I.D. stainless steel, packed with LiChrosorb RP-8, mean particle diameter 10 μm. Injections were made using a Valco 7000 p.s.i. injection valve, fitted with a 10-μl sample loop.

RESULTS AND DISCUSSION

The method for the determination of saccharin, caffeine and benzoic acid as described by Smiley *et al.*[10] gives impractically long retention times. The method uses a 6% acetic acid solution in water as the mobile phase. It was decided to use a simple simplex method[11], with analysis time as optimizing criterion, to locate a suitable region in which to situate the lattice design. Obviously the simplex tended to climb to higher organic solvent concentrations (Fig. 2), but the experiments involved at this stage gave valuable information over an extended region of the factor space. From these experiments it could be concluded that the resolution between both pairs of adjacent peaks approached 1.00 at approximately 60% of organic solvent. As the simplex favoured acetonitrile for shorter analysis times, a lattice design was located as indicated in Fig. 2. The results are given in Table I, and were used to calculate via polynomial regression the following polynomial equations:

$$T = -5.0013x_1 + 16.152x_2 + 8.802x_3 + 4.7564x_1x_2 - 2.7436x_1x_3 - 37.9756x_2x_3 \tag{1}$$

$$R_{12} = -11.1545x_1 + 2.7164x_2 + 0.9755x_3 + 18.5754x_1x_2 - 11.1676x_1x_3 - 2.5528x_2x_3 \tag{2}$$

$$R_{23} = -0.3792x_1 + 8.808x_2 + 2.4705x_3 - 2.9774x_1x_2 - 2.5573x_1x_3 - 17.1378x_2x_3 \tag{3}$$

where x_1 = volume fraction of methanol, x_2 = volume fraction of water, x_3 = volume fraction of acetonitrile and T = analysis time; for R_{12} and R_{23}, see Table I.

These expressions were used to calculate and plot the response surfaces in Fig. 3, where the constraint boundaries are also drawn in. It is possible to determine the

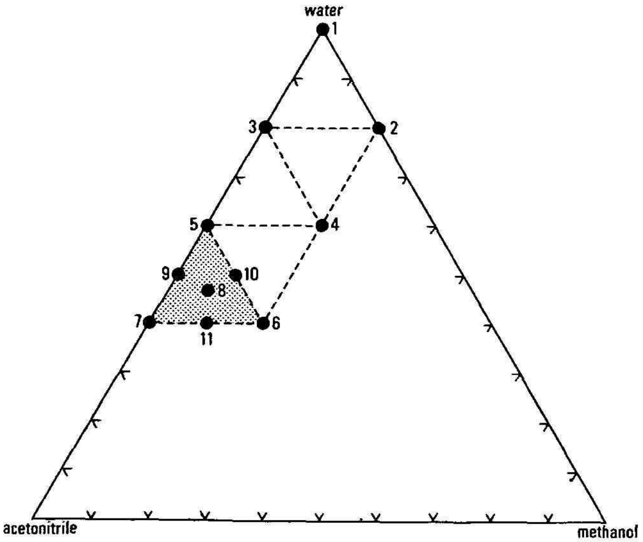


Fig. 2. Progress of simplex algorithms during optimization. Shaded area is the region where the simplex lattice design was located (points 5–11 from Table I).

TABLE I

ANALYSIS TIME AND RESOLUTIONS MEASURED BETWEEN SACCHARIN AND CAFFEINE (R_{12}) AND CAFFEINE AND BENZOIC ACID (R_{23}) (SEE ALSO FIG. 2)

Experiment No.	*Volume fraction*			R_{12}	R_{23}	*T (min)*
	Water	*Methanol*	*Acetonitrile*			
1	1.00	0.00	0.00	>2	>2	>30
2	0.80	0.20	0.00	>2	>2	>20
3	0.80	0.00	0.20	1.96	4.80	10.13
4	0.60	0.20	0.20	2.00	3.19	6.50
5	0.60	0.00	0.40	1.36	1.82	4.07
6	0.40	0.20	0.40	1.19	1.24	3.06
7	0.40	0.00	0.60	0.94	0.88	2.63
8	0.466	0.066	0.466	1.47	1.27	3.08
9	0.50	0.00	0.50	1.33	1.40	3.00
10	0.50	0.10	0.40	1.29	1.70	3.66
11	0.40	0.10	0.50	1.23	1.02	2.85

optimum analysis time graphically from Fig. 3. Suppose one requires a minimum resolution of 1.25, corresponding to 99.8% resolution of the peaks in the chromatogram. From the countour lines of the analysis time, one sees that increasing the amount of organic solvent in the mobile phase decreases the analysis time. However, compositions beneath the isocontour lines that indicate resolutions of 1.25 are not allowed (shaded area). It can easily be seen that the minimum analysis time, while maintaining a resolution of 1.25, is the intersection of the two constraints. From this the optimum composition is seen to be water–methanol–acetonitrile = 0.47:0.03:0.50, where the analysis time can be calculated from eqn. 1, its value being 2.94 min.

If a minimum resolution of 1.5 is required, the graphical approach is less suitable. The constraint will be met by the resolution between saccharin and caffeine becoming 1.5, the resolution between caffeine and benzoic acid still being far better than 1.5, and of no concern. The optimum can be located by finding the smallest distance between the constraint line and the next lower contour line of the analysis time. This is a tedious procedure, however, and it is possible to calculate the optimum from eqns. 1, 2 and 3 mathematically. Calculation of the optimum is also recommended when the number of peaks is so great that the contour plots become difficult to read. Once the models 1, 2 and 3 have been calculated, the problem of finding the minimum analysis time while maintaining a resolution of at least 1.5 can be stated as follows: Minimize

$$T = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

subject to the following constraints:

$$x_1 + x_2 + x_3 = 1$$
$$x_1 \geqslant 0$$
$$x_2 \geqslant 0$$
$$x_3 \geqslant 0$$
$$R_{ij} \geqslant \alpha_{1,ji} x_1 + \alpha_{2ji} x_2 + \alpha_{3,ji} x_3 + \alpha_{12,ji} x_1 x_2 + \alpha_{13,ji} x_1 x_3 + \alpha_{23,ji} x_2 x_3$$

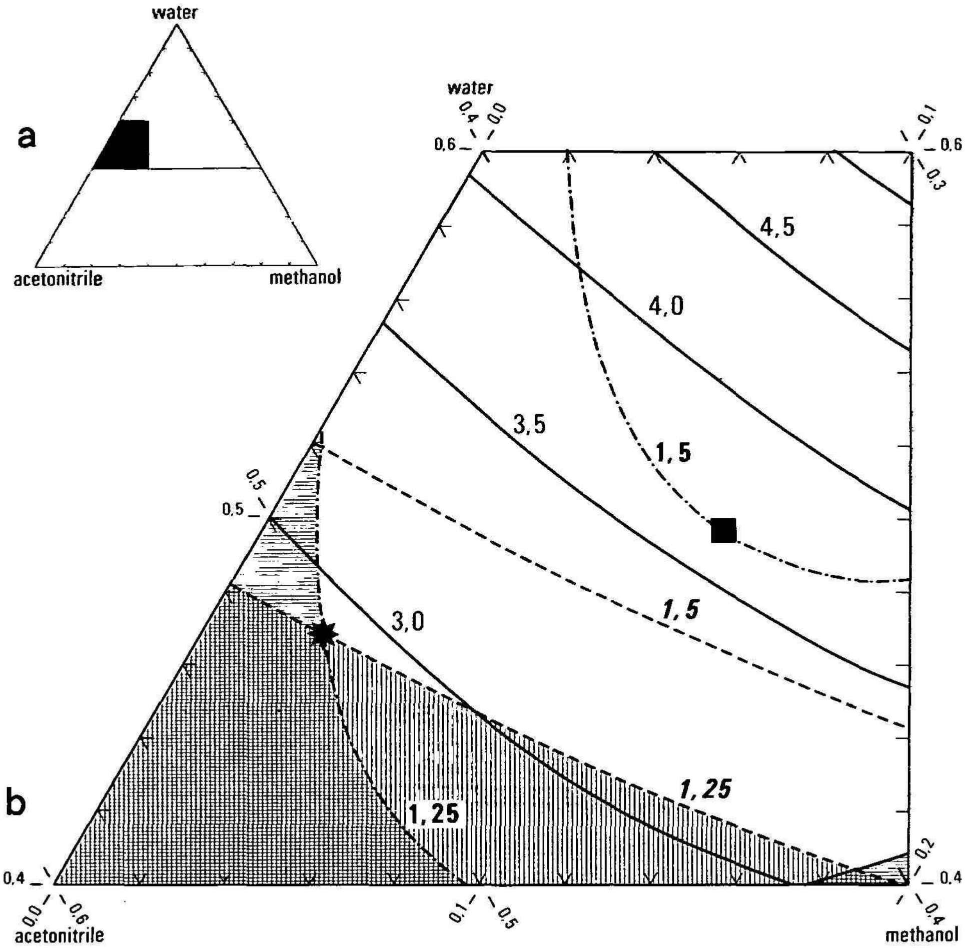


Fig. 3. (a) Accessible part of the factor space (shaded region) and part of the factor space shown in (b) (black region). (b) Contour plots over the region of the factor space indicated in (a): (———) analysis time; (-·-·-·-·-) resolution between saccharin and caffeine; (--------) resolution between caffeine and benzoic acid). The shaded areas are outside the constraints for resolution 1.25. Optimum compositions for minimum resolution 1.25 denoted by ★, and for 1.5 by ■.

for $i = 1, n$ and $j = i + 1, n$ (R_{ji} = resolutions between each pair of peaks).

In our specific case there are only two resolutions to be considered, because in the region in which we are interested the elution sequence does not change. It is necessary to take into account only the separation between adjacent peaks, and not between peaks that have no overlap in the factor region.

The solution of this problem, minimize a non-linear objective function, subject to non-linear inequality constraints, is possible by an operations research technique called non-linear programming. An explanation of this technique is beyond the scope of this paper, but any good operational research textbook will provide a thorough discussion of the method[12]. The calculated solution to the stated non-linear programming problem is given in Table II for constraints of 1.25 and 1.5 on the resolution (R).

TABLE II

COMPARISON OF EXPERIMENTAL RESULTS WITH PREDICTIONS FROM THE MODEL AT CALCULATED OPTIMUM ELUENT COMPOSITIONS FOR $R_m > 1.25$ AND $R_m > 1.5$

Calculated optimum composition			*Calculated time (min)*	*Measured time (min)*	$R_{1,2}$ *constraint*	$R_{1,2}$ *measured*	$R_{2,3}$ *constraint*	$R_{2,3}$ *measured*
Water	*Methanol*	*Acetonitrile*						
0.470	0.027	0.503	2.93	2.94	1.25	1.18	1.25	1.29
0.496	0.108	0.396	3.63	3.23	1.50	1.42	1.50	1.80

The calculated optimum is found to be in good agreement with the optimum obtained graphically. Also summarized in Table II are the results of experiments carried out at the optimum composition. These results confirm the calculated predictions from the model with regard to analysis time and resolution well within experimental error; Fig. 4 shows chromatograms recorded during the progress of the simplex and at optimum composition. It appears that the method described is suitable for practical optimizations in chromatography. A final remark should be made. It is possible to substitute

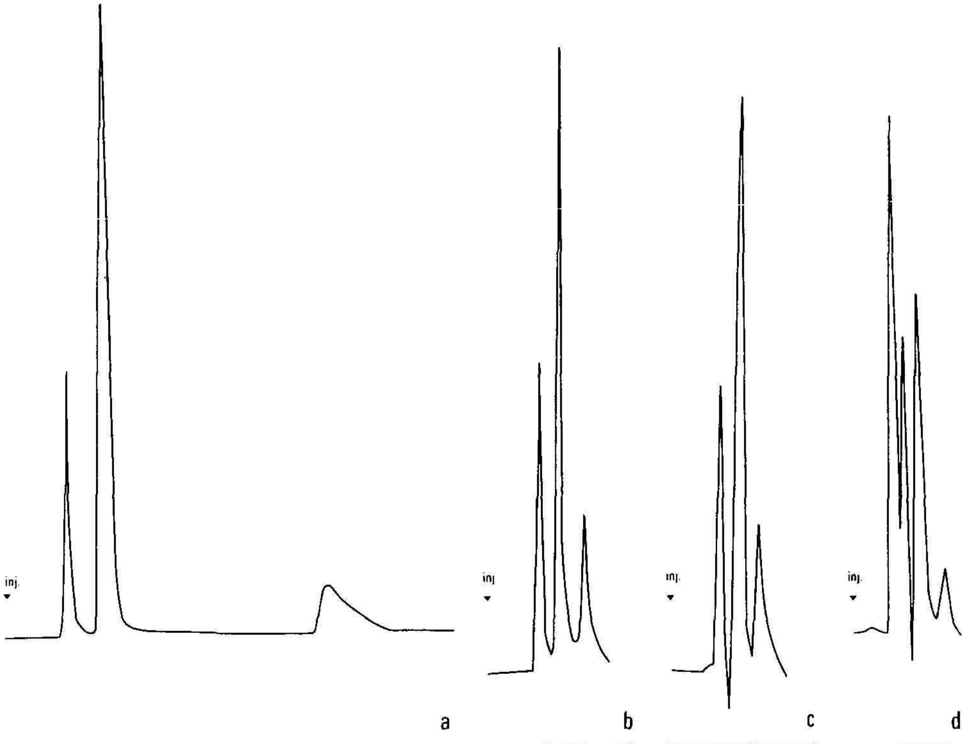


Fig. 4. Chromatograms of the standard mixture recorded during the optimization (a,b) and at optimum composition for R = 1.25 (c). Elution sequence: saccharin, caffeine, benzoic acid. (d) Chromatogram of dietary Cola at optimum composition. Eluent composition: water–methanol–acetonitrile = (a) 0.80:0.00:0.20, (b) 0.466:0.066:0.466, (c) and (d) 0.47:0.03:0.50 (optimum for R = 1.25).

cost per analysis for analysis time as the optimizing criterion, in which case probably a different optimum would have been found, trading off time against eluent cost.

REFERENCES

1 L. R. Snyder, in A. Weissberger and E. S. Perry (Editors), *Separation and Purification, Techniques of Chemistry*, Vol. 3, Part 1, Wiley-Interscience, New York, 2nd ed., 1978, Ch. 2.
2 L. R. Snyder, *J. Chromatogr.*, 92 (1974) 223–230.
3 J. H. Hildebrand, J. M. Prausnitz, and J. M. M. Scott, *Regular and Regulated Solutions*, Van Nostrand Rheinhold, New York, 1970.
4 R. Tijssen, H. A. H. Billiet and P. J. Schoenmakers, *J. Chromatogr.*, 122 (1976) 185–203.
5 P. H. J. Schoenmakers, *Ph.D. Thesis*, Technical University, Delft, 1981.
6 J. W. Gorman and J. E. Hinman, *Technometrics*, 4 (1962) 4.
7 R. D. Snee, *Chemtech*, 9 (1979) 702–710.
8 B. R. Belinky, *Occupational Health Chemistry*, ACS Symposium Series No. 120, American Chemical Society, Washington, DC, 1980, pp. 149–168.
9 J. L. Glajch, J. J. Kirkland and K. M. Squire, *J. Chromatogr.*, 199 (1980) 57–79.
10 D. S. Smiley, B. Woodward and E. C. Conrad, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 14.
11 J. A. Nelder, R. Mead, *Comput. J.*, 7 (1965) 308.
12 T. Phillips, *Operations Research, Principle and Practice*, Wiley-Interscience, New York, 1976.

Journal of Chromatography, 247 (1982) 231–243
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,064

DIRECT FLUID INJECTION INTERFACE FOR CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY–MASS SPECTROMETRY*

RICHARD D. SMITH*
*Chemical Methods and Kinetics Section, Physical Sciences Department, Pacific Northwest Laboratory**, Richland, WA 99352 (U.S.A.)*
and
JOHN C. FJELDSTED and MILTON L. LEE
Department of Chemistry, Brigham Young University, Provo, UT 84602 (U.S.A.)

(First received March 26th, 1982; revised manuscript received May 24th, 1982)

SUMMARY

A new interface for capillary column supercritical fluid chromatography–mass spectrometry (SFC–MS) is described and initial results are presented. The advantages of SFC include the ability to separate high-molecular-weight, non-volatile and thermally unstable compounds not amenable to gas chromatography. Capillary column SFC–MS has potential advantages over high-performance liquid chromatography–MS owing to the higher possible chromatographic efficiency, mobile phase volatility and the simplicity of the interface design. The direct fluid injection interface provides for transfer of the total capillary SFC effluent into a chemical ionization source. Initial results are presented to illustrate the separation and analysis of simple mixtures of aromatic hydrocarbons and styrene oligomers using *n*-pentane as the mobile phase.

INTRODUCTION

A mass spectrometer constitutes a nearly ideal detector for chromatography, as evidenced by the explosive growth in the application of gas chromatography–mass spectrometry (GC–MS) in the last decade. The success of this approach and the range of samples not amenable to GC–MS spurred the development of high-performance liquid chromatography (HPLC)–MS. However, interfacing conventional packed column HPLC to mass spectrometry has been a difficult task because of the incompatibilities in required liquid flow-rates and solvent evaporation or removal[1–3]. While significant progress has been made in the development of various LC–MS interfaces, these methods have not approached the routine application[4,5] of GC–MS instrumentation. Hence, there is a clear need for an alternative approach to HPLC for the analysis of complex mixtures of non-volatile compounds providing increased chromatographic efficiency as well as greater compatibility with mass spectrometry.

---

* Prepared for the U.S. Department of Energy under Contract DE-AC06-76RLO 1830.

** Operated by Battelle Memorial Institute.

Supercritical fluid chromatography (SFC) has developed over the past two decades as an alternative to HPLC with specific advantages in some applications[6–10]. The combination of SFC with mass spectrometry may also provide advantages over HPLC–MS. The range of compounds which may be separated with SFC is similar to that with HPLC, although the state-of-the-art in HPLC is considerably more advanced than in SFC. Both techniques allow the separation of materials which are thermally labile and of much higher molecular weight than is possible using GC[6–10]. SFC has the added capability of pressure programming, providing precise control of the solvating power of the mobile phase and an alternative to gradient elution in HPLC. Additionally, the much higher volatility of most SFC mobile phases provides a significant advantage in the design of the SFC–MS interface. These advantages were recognized by Randall and Wahrhaftig[11–13], who have previously reported on the construction of a supercritical fluid (dense gas) chromatograph–mass spectrometer interface using conventional packed columns and supersonic molecular beam techniques. This approach, however, suffered from low sensitivity, the complexity of four stages of differential pumping required by the large mobile phase flow-rates and mass spectra that were complicated by cluster formation in the beam expansion[13].

This paper describes the development of an interface for capillary column SFC–MS. The development of capillary column SFC has recently been reported[14–16]. The use of wall-coated open-tubular capillary columns provides mobile phase flow-rates compatible with chemical ionization mass spectrometry and removes the need for a liquid–gas phase transition as in HPLC–MS. Other advantages accrue from the high chromatographic efficiency projected for capillary columns owing to the low pressure drop across the column and the more uniform density of the mobile phase[14–16]. This work demonstrates the compatibility of this technique with mass spectrometry and has demonstrated sensitivities and separations comparable to or better than HPLC–MS.

EXPERIMENTAL

The emphasis of our work has been (a) the development of a working capillary SFC–MS interface, (b) determination of parameters for an optimum interface design and (c) an investigation of the range of application. Fig. 1 illustrates the SFC–MS instrument constructed in this work. The instrument incorporates a high-pressure programmable pump, a constant-temperature oven, capillary column, the direct fluid injection (DFI) interface and a tandem quadrupole mass spectrometer equipped with a dual electron impact–chemical ionization ion source.

The supercritical fluid chromatograph utilizes a Varian 8500 high-pressure syringe pump (8000 p.s.i. maximum pressure) and a constant-temperature oven and transfer line. Fused silica capillary columns, of 100- or 200-μm I.D. and lengths of 10–30 m, were used in our initial studies.

Capillary columns were prepared by immobilizing the stationary phase through peroxide cross-linking of SE-52 and SE-54[17] in a manner similar to that described by Grob *et al.*[18,19]. At present the selection of mobile phases is limited by the range of columns that have been prepared for capillary SFC, restricting the use of more polar mobile phases. The injection–splitter assembly allows reproducible 0.2-μl injections[16], although the precise splitting ratio is not known. A Hewlett-Packard GC

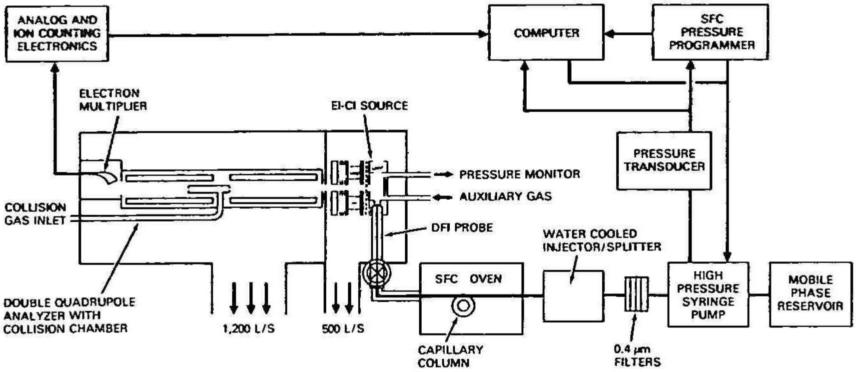


Fig. 1. Overall schematic diagram of the capillary column SFC–MS system.

temperature programmer was modified to provide pressure programming of the syringe pump[20]. In our work to date isobutane and *n*-pentane have been used as mobile phases. Most work has utilized *n*-pentane. Alternative mobile phases, such as carbon dioxide[9], offer distinct advantages for the analysis of thermally labile compounds owing to the lower critical temperatures. Column temperatures slightly above the critical temperatures (147°C for isobutane where T_c = 135°C, and 210°C for *n*-pentane where T_c = 196°C) are typically used to reduce the effects of thermal variations upon the mobile phase[10]. Pressure programs ranging from 20 to 75 atm for *n*-pentane were used in this work.

The principal design considerations for the DFI probe involved obtaining constant-temperature conditions, proper column restriction to obtain optimum flow-rates and efficient transfer of non-volatile compounds to the ionization region.

Optimum chromatographic resolution in SFC requires maintaining a constant temperature over the entire length of the column and elimination of all dead volumes between the column and the detector. The present instrument attains these criteria by extending the fused silica column through a constant temperature (±0.4°C) transfer line to within 3 cm of the DFI probe tip. The DFI probe contains separate heating elements to maintain the required temperature conditions. The fused silica capillary column is connected to the DFI probe tip by a zero dead volume connection (fabricated from a short length of 300-μm I.D. tubing) to 100-μm I.D. (300-μm O.D.) platinum–iridium tubing. A silver chloride melt is used as a sealant. The platinum–iridium tubing extends through the last 3 cm of the interface to the flow restrictor which determines the mobile phase flow-rate.

Fig. 2 illustrates two of the probe designs we have evaluated. The probe illustrated in Fig. 2A employs a laser-drilled orifice as a pressure restrictor. A 0.5–2.0-μm hole is drilled in 13-μm thick stainless steel. A small tin gasket is used to make a tight seal between the probe tip and the pressure restrictor, resulting in a dead volume estimated to be of the order of 0.01 μl. The second DFI probe design (Fig. 2B) is similar but terminates in a 0.2–0.5-mm length capillary restriction. This restriction is formed, in this instance, by crimping the tubing termination to obtain the desired flow-

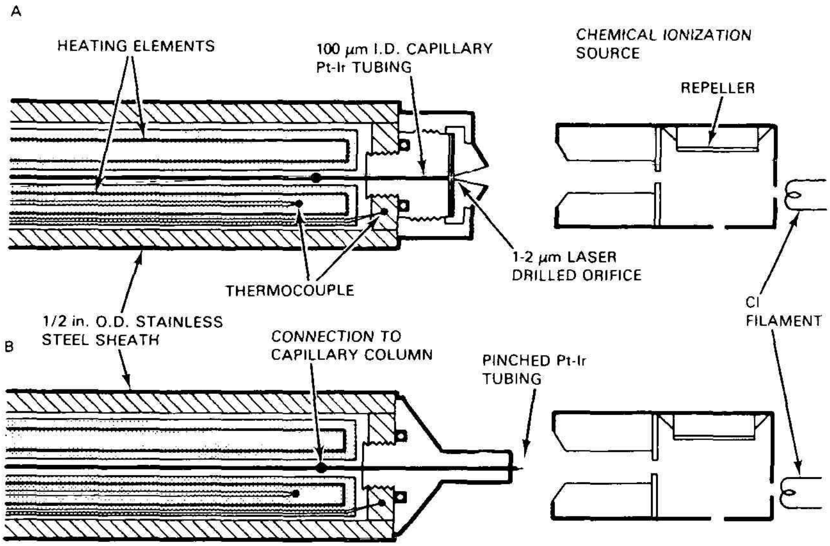


Fig. 2. Schematic diagram of two of the direct fluid injection probes designed for interfacing SFC with a conventional CI ion source: (A) a probe using a non-viscous laser-drilled orifice restrictor; (B) probe utilizing a pinched (< 1-mm) capillary restrictor.

rate. This restriction also provides an effective zero dead volume. All probe designs are operated at the SFC temperature to obtain optimum chromatographic resolution. The two probes have given similar performance in our initial studies; however, the orifice in the first design appears to plug more readily. Probe design considerations are discussed further in the next section. Additional probe designs of combined characteristics are also being evaluated.

The DFI probes have been designed to couple with the direct probe inlet of a "simultaneous" chemical ionization–electron impact (CI–EI) Extranuclear Labora-

TABLE I

FLOW-RATES AND MASS SPECTROMETER CHAMBER PRESSURES

Typical operating conditions.

Property	*Column diameter (mm)*	
	0.1	*0.2*
Linear velocity (cm/sec)	3.0	2.2
Flow-rate (as liquid) (μl/min)*	20	60
SFC pressure (atm)	28	28
Chemical ionization source pressure (torr)	0.3	1.0
Ion source chamber pressure (torr)	$3 \cdot 10^{-4}$	$1.0 \cdot 10^{-3}$
Mass spectrometer chamber pressure (torr)	$0.8 \cdot 10^{-7}$	$2.5 \cdot 10^{-6}$

* Assuming the stated column diameter is available to mobile phase flow (*i.e.*, neglecting the contribution of the stationary phase wall coating).

tories (Pittsburgh, PA, U.S.A.) ion source. A second CI source inlet provides additional chemical ionization reagent gas (usually the SFC mobile phase). A Granville-Phillips servo-valve and a pressure controller are used to regulate gas flow. Thus, a constant CI source pressure is maintained during SFC pressure programming where the flow to the ion source changes by as much as a factor of 2–3. The ion source chamber is pumped with a 500 l/sec turbomolecular pump and the mass spectrometer chamber is pumped with a 4-in. diffusion pump at a nominal pumping speed of 1200 l/sec. Table I gives the flow-rates, ion source and mass spectrometer chamber pressures for reasonable flow-rates using 0.1- and 0.2-mm I.D. columns and *n*-pentane as the mobile phase[16].

The mass spectrometer is an Extranuclear Laboratories tandem quadrupole mass filter, providing collision-induced dissociation (CID) capability[21]. The mass spectrometer has a mass range of m/z 3–1400, with computer-controlled data acquisition and storage.

RESULTS AND DISCUSSION

DFI probe and interface design

The successful interfacing of a capillary column SFC to a mass spectrometer requires stable SFC conditions, minimizing or eliminating dead volumes and providing the necessary pressure restriction. The present interface design provides for the necessary constant-temperature conditions and essentially eliminates all dead volumes.

The design of the DFI probe tip restrictor is a critical factor in SFC–MS performance, as in LC–MS[22–24]. An ideal restrictor will provide the desired restriction and mobile phase flow-rate, will maintain supercritical conditions to as close to the probe tip as possible and will provide a jet of vapors directed into the chemical ionization volume. Additionally, the interface to the CI source should provide for minimization of solvent clusters and eliminate or reduce the condensation or decomposition of non-volatile or thermally labile compounds. The interface should also provide flexibility in the choice of CI reagent gases and provide a flow-rate compatible with CI. Finally, the interface should be simple, easily maintained and capable of prolonged operation without failure (*e.g.*, plugging of the restrictor).

The properties of supercritical fluids allow the capillary column DFI interface to approach these requirements more closely than with conventional packed column LC–MS coupling. Direct liquid injection LC–MS interfaces deliver approximately 1–4% of the total effluent into the mass spectrometer ion source[22–24], although more extreme splitting ratios have been reported by some workers. Indeed, more recent work with micro packed columns has demonstrated the injection of the total LC effluent[25]. The principal problems with this interface result from the low volatility of most solvents and the effects upon ion source operation with mobile phases such as water. Additionally, cooling of the probe and orifice has been found desirable in HPLC–MS operation to prevent solvent volatilization (which results in large changes in introduction rate and the loss, or condensation, of material near the orifice). Some of these difficulties may be avoided using an extended capillary restrictor as reported by Krien *et al.*[26]; however, it has not been demonstrated that this approach avoids the predicted problems due to volatilization in the capillary and deposition of non-volatile compounds.

The capillary SFC–MS interface avoids problems related to solvent evaporation by maintaining supercritical (dense gas) conditions up to the DFI restrictor. Proper design of the injector tip can allow a rapid expansion of the dense gas avoiding the liquid–gas phase transition in LC–MS. Additional advantages result from the much higher volatility of typical mobile phases compared with HPLC, where typical conditions result in extensive cluster formation and persistence of a jet of frozen liquid droplets extending 2–5 cm into the vacuum region[24]. In the DFI interface the *n*-pentane jet disappears after less than 0.5 cm for flow-rates as large as 100 μl/min. More typical flow-rates show no visible jet. A result of the mobile phase volatility is that the mass spectra show no evidence of ion clusters. Similar results were obtained for isobutane and can be expected for other mobile phases. Preliminary results with carbon dioxide as a CI reagent indicate that these more advantageous mobile phases are compatible with both positive and negative ion CI, suggesting significant advantages upon development of more adequate bonded stationary phases.

It is clear that the most important single component in the DFI interface is the restrictor. The restrictor is chosen to provide the desired flow-rate. Owing to the complex nature of the flow under supercritical conditions, the final design of acceptable restrictors is necessarily approached empirically. We have examined both short viscous (*i.e.*, capillary) and more nearly non-viscous (laser-drilled orifice) restrictors.

The use of extended viscous restrictors above the critical temperature is clearly undesirable, as the gradual pressure drop will lower the mobile phase density gradually and lead to solute deposition within the capillary tube. The use of an extended viscous restrictor in conjunction with cooling to below the critical temperature causes two phase changes and presents additional problems. As the dense gas condenses a liquid results in which some components may not be soluble. This is followed by an additional loss of non-volatile materials as the solvent evaporates at the probe tip[24].

The use of very short (<1 mm) viscous and more nearly non-viscous restrictors results in a small jet of submicron droplets that disappears within <0.5 cm of the restrictor for typical flow-rates (<100 μl/min). The use of small laser-drilled orifices in 13-μm thick stainless steel provides a reasonably non-viscous restriction. The ideal orifice diameter size is *ca.* 0.5 μm for 0.1-mm I.D. columns and *ca.* 1.0 μm for 0.2-mm I.D. columns. The major difficulties involve selection of the orifice (as the production of uniform orifices in this size range is difficult) and possible plugging of the orifice. This is particularly a problem for the smaller orifice sizes (<1.0 μm). Care must also be taken to minimize the restrictor surface area subjected to the high pressure, as these thin diaphragms are easily deformed, resulting in an increased orifice size and causing an additional dead volume.

These problems are substantially reduced by using very short viscous restrictors. This design avoids the phase changes associated with long capillary restrictors and allows a larger orifice. Crude restrictors of this type have been made by carefully pinching the termination of 0.1-mm I.D. platinum–iridium tubing to provide the desired flow and jet characteristics. These restrictors have been demonstrated to function for several days before plugging and mass spectra confirm efficient transfer to the CI source of non-volatile compounds, similar to non-viscous restrictors. Research into the development of reproducible and easily replaced restrictors is continuing.

DFI interface operation

The capillary SFC–MS interface is a reliable and simple device. The interface has been designed to use a direct insertion interlock for easy removal and maintenance of the DFI probe.

Typical operating procedures that have proved successful involve first starting the SFC flow prior to probe introduction with the oven and transfer line at proper temperature (210°C for *n*-pentane) and with the probe at ambient temperatures. After insertion into the vacuum the SFC pressure is increased to 20–30 atm. The flow-rate is evaluated using the ion source chamber pressure as a guide. The DFI probe temperature is then increased to 210°C. The entire start-up period requires less than 10 min.

The CI source pressure due to SFC flow ranges from 0.1 to 2 torr depending on the column diameter and the desired flow-rate. A single SFC separation may involve pressure programming over a range of 20–60 atm and result in a corresponding change in ion source pressure. As optimum ion source tuning is dependent on pressure, the CI ion source pressure was held constant using an auxiliary gas inlet and servo-valve to provide optimum and constant chemical ionization conditions regardless of column pressure. This is particularly important for 0.1-mm I.D. columns where flow-rates are small and additional gas is necessary to optimize sensitivity. The effect of CI pressure is illustrated in Fig. 3 for multiple injections of pyrene on a short 1-m column. Mass spectra were obtained for thirteen 0.2-μl injections of a 1:1000 solution in *n*-pentane, corresponding to 200 ng per injection before the splitter. Fig. 3 gives the reconstructed ion chromatogram for the $(M + 1)^+$ ion (m/z 203). Ion source pressures of 0.4 torr were obtained for the first injection and the pressure was increased in

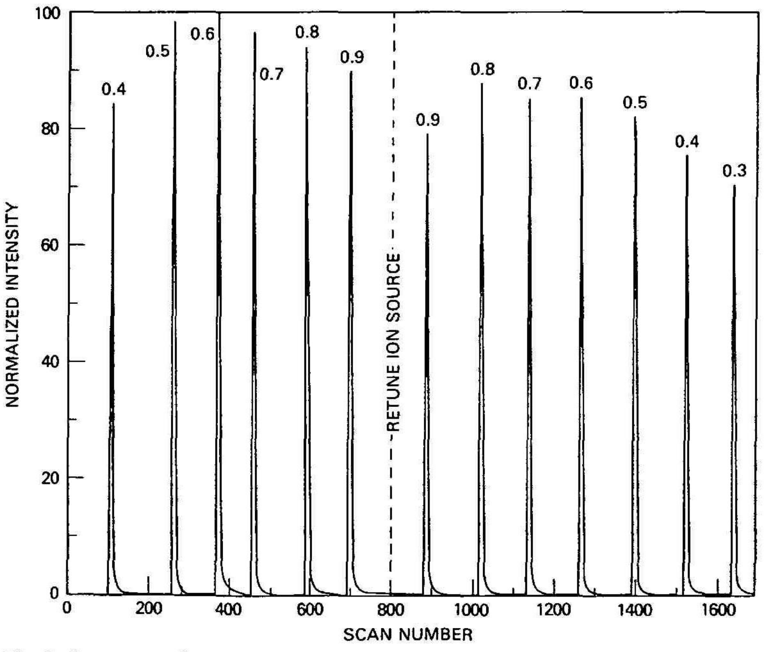


Fig. 3. Ion current for m/z 203 for a series of identical pyrene injections at various ion source pressures (in torr). The ion source was tuned at 0.4 torr and left unchanged for five subsequent injections at higher pressures and then retuned at 0.9 torr for the subsequent injections.

0.1-torr increments using the auxiliary gas inlet for the next five injections without retuning the ion source and ion optics voltages. The ion source was then retuned and pressures were decreased in 0.1-torr increments. These results demonstrate that the effects of pressure changes are relatively minor and that optimum sensitivity is obtained for approximately 0.6–0.8 torr pressure in our ion source. If constant CI source pressure is maintained using the auxiliary inlet, then the mass spectrometer response (peak area) is independent of flow-rate (SFC pressure) and the variation in response results from the injector operation (approximately $\pm 5\%$).

The chemical ionization source provides both excellent sensitivity and flexibility owing to the potential for the addition of different CI reagent gases. For 100-μm I.D. columns at optimum column flow-rates the added gas can amount to as much as 90% of the CI reagent gas. This allows the capability of mixed CI reagent gases and eases limitations due to the ionization method. The two CI reagents used to date, isobutane and *n*-pentane, provide "mild" ionization conditions owing to the dominant proton transfer process. Many compounds analyzed in this manner yield spectra with a dominant $(M + 1)^+$ ion and little additional fragmentation, although for *n*-pentane $(M + 43)^+$, $(M + 55)^+$ and $(M + 71)^+$ peaks, often with distinctive

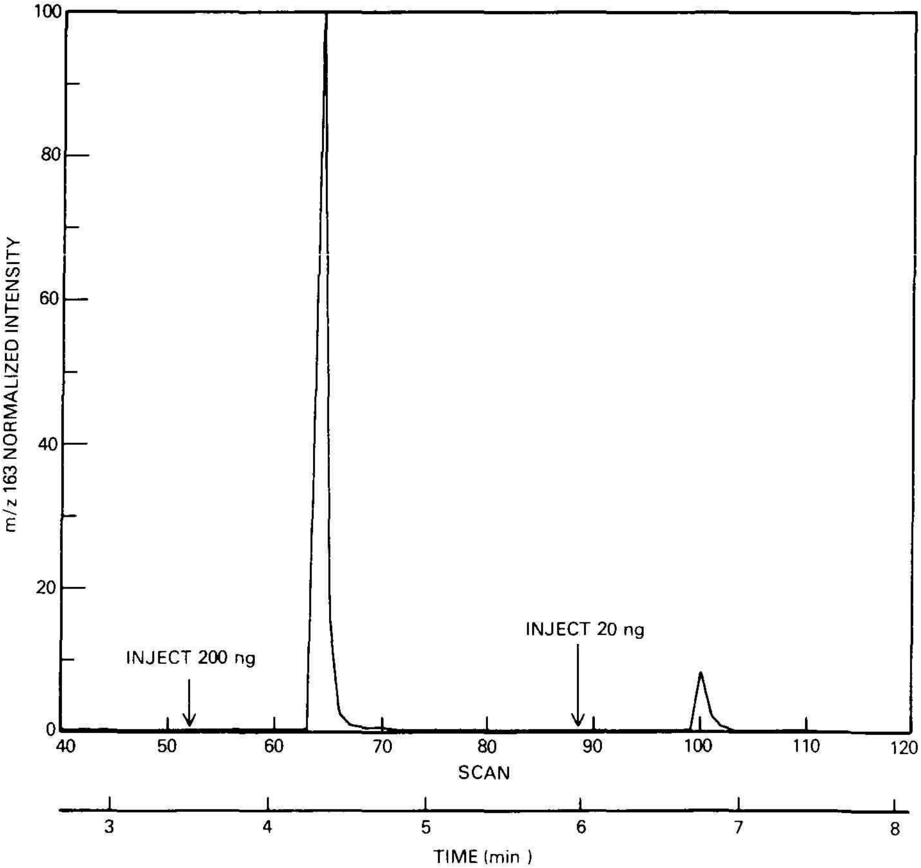


Fig. 4. Reconstructed single ion chromatogram for m/z 163, the $(M + 1)^+$ ion for triethylbenzene. Plot shows the response for two injections of 200 and 20 ng.

relative intensities, are also observed for some compounds. For cases where additional information is necessary for identification one can use either more energetic mixed CI reagents or the CID capability of the double quadrupole analyzer[21].

Fig. 4 illustrates the response obtained for two triethylbenzene injections of 200 and 20 ng, respectively, for a flow-rate of *ca.* 17 cm/sec (*ca.* 30 μl/min of liquid) using a 0.1-mm I.D. column. The mass spectra show a dominant $(M + 1)^+$ ion at m/z 163 to constitute the base peak and no other ions amounting to more than 10% of m/z 163. These results obtained while scanning the mass spectrometer illustrate the excellent sensitivity of the technique even with overall decreased ion currents resulting from the use of a tandem quadrupole mass filter and an uncertain injector splitting ratio. The signal-to-noise ratio for the $(M + 1)^+$ ion is greater than 100 for 20-ng injections, suggesting detection limits in the subnanogram range while scanning and in the low picogram range for single ion monitoring even for broad peaks for compounds with long retention times. Owing to the decreased splitting ratio, at present greater sensitivity is obtained using 0.2-mm I.D. columns while improved chromatographic resolution can be obtained with 0.1-mm I.D. columns[16].

Fig. 5 illustrates the application of the capillary SFC–MS to a mixture of five polycyclic aromatic hydrocarbon compounds in methylene chloride. The separation used a 20 m × 0.2 mm I.D. column and *n*-pentane as the mobile phase. The injected mixture contained a nominal 100 ng of each component. The reconstructed total ion chromatogram shows that the mixture of naphthalene, anthracene, pyrene,

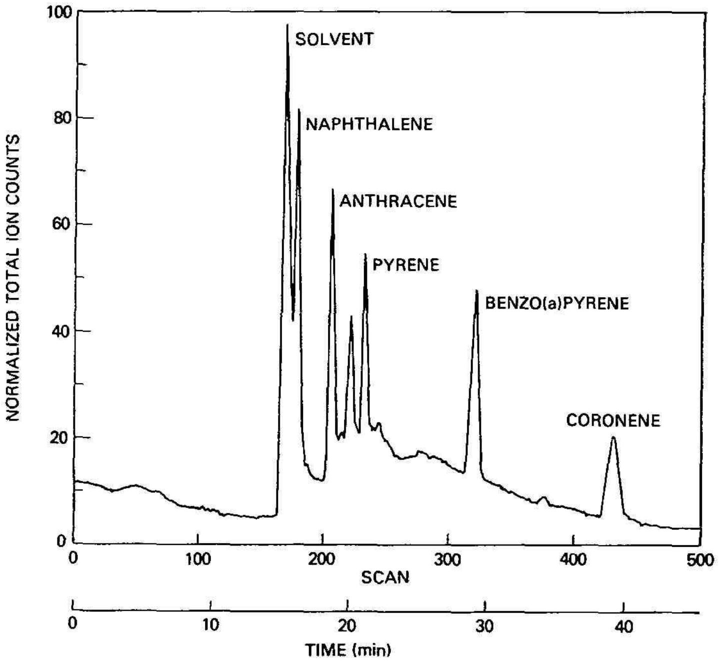


Fig. 5. Total reconstructed ion chromatograph for a SFC–MS analysis of a mixture of five polycyclic aromatic hydrocarbons and associated impurities. The separation used a 20 m × 0.2 mm I.D. column and a pressure program involving isobaric conditions at 24 atm for the first 15 min followed by a 0.8 atm/min increase to 40 atm.

benzo[*a*]pyrene and coronene was easily separated. The solvent peak actually results from response due to increased solubility of material on the column or a slight stripping of the column resulting from the use of methylene chloride solvent. Fig. 5 also shows evidence of several additional impurities. Fig. 6 gives reconstructed single ion chromatograms for eight $(M + 1)^+$ ions for compounds in the mixture, including three impurities having molecular weights of 208, 226 and 276. Evidence has been obtained for at least 20 additional minor impurities in this mixture. Fig. 7 gives the mass spectra obtained for anthracene, pyrene and benzo[*a*]pyrene in this

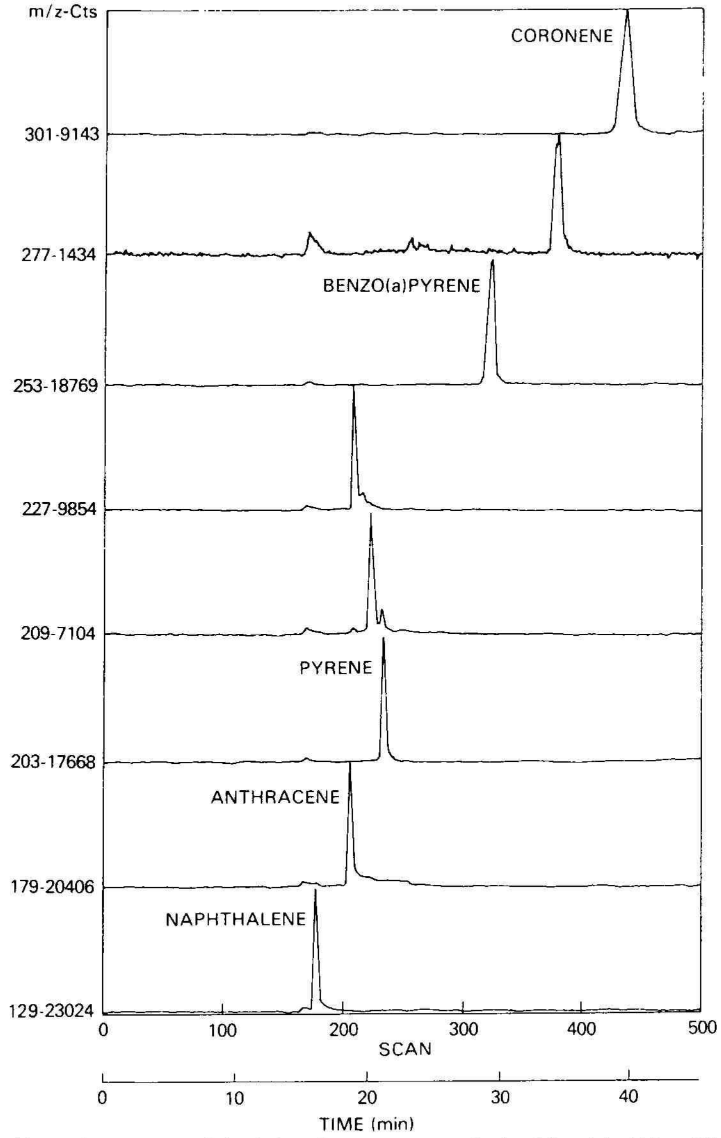


Fig. 6. Reconstructed single ion chromatograms obtained for eight $(M + 1)^+$ ions in the separation shown in Fig. 5. Also given are the $(M + 1)^+$ chromatograms for three impurities having molecular weights of 208, 226 and 276.

separation. The spectra show minor contributions of $(M + 43)^+$, $(M + 55)^+$ and $(M + 71)^+$ ions in most cases. The anthracene spectrum also shows a sizable impurity of molecular weight 226 which elutes simultaneously. These spectra demonstrate that high quality mass spectra can be obtained during capillary SFC–MS analyses.

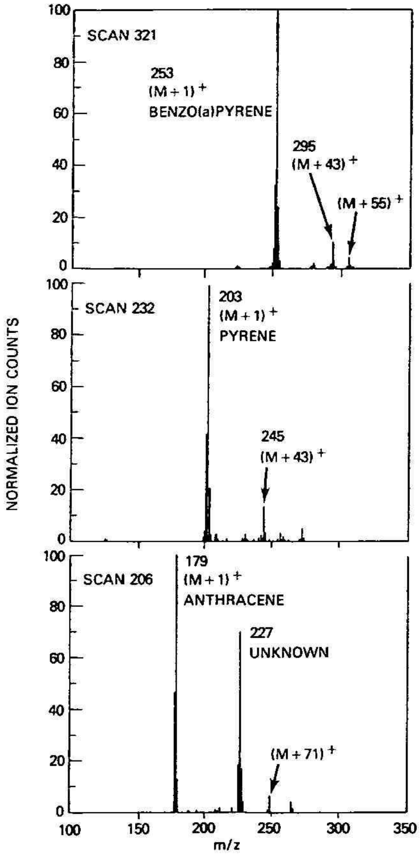


Fig. 7. Mass spectra for three of the peaks in the chromatogram given in Fig. 5. The mass spectra show the presence of $(M + 43)^+$, $(M + 55)^+$ and $(M + 71)^+$ ions for some compounds. Scan 206 contains evidence of a large impurity eluting nearly simultaneously with anthracene (see Fig. 6).

The application of the SFC–MS instrument to non-volatile high-molecular-weight compounds is illustrated in Fig. 8, which gives the total reconstructed ion chromatogram for a separation of a polystyrene sample having a nominal molecular weight of 800. The separation used a 0.2-mm I.D. column, a programmed pressure increase from 28 to 60 atm and *n*-pentane as the mobile phase. The chromatogram shows the first twelve styrene oligomers to be clearly resolved. The molecular weight of the individual oligomers is $104n + 58$; thus the twelfth oligomer has a molecular weight of 1306. The mass spectra show $(M + 1)^+$ ions and distinctive fragment ions for each oligomer, clearly proving the separation and demonstrating that the mass spectra are obtained without detectable decomposition of the individual oligomers. Additionally, the largest peak areas are obtained for the $n = 7$ and $n = 8$ oligomers as would be expected from the known average molecular weight. These results demonstrate the efficient transfer of compounds (*i.e.*, the larger oligomers) expected to be non-volatile for the ion source temperature of 210°C. Although similar separations can be obtained using HPLC, these results demonstrate that capillary column SFC is a potentially powerful alternative technique. Similar results have been obtained for other essentially non-volatile compounds, demonstrating that such compounds can be efficiently transferred to the ion source and ionized.

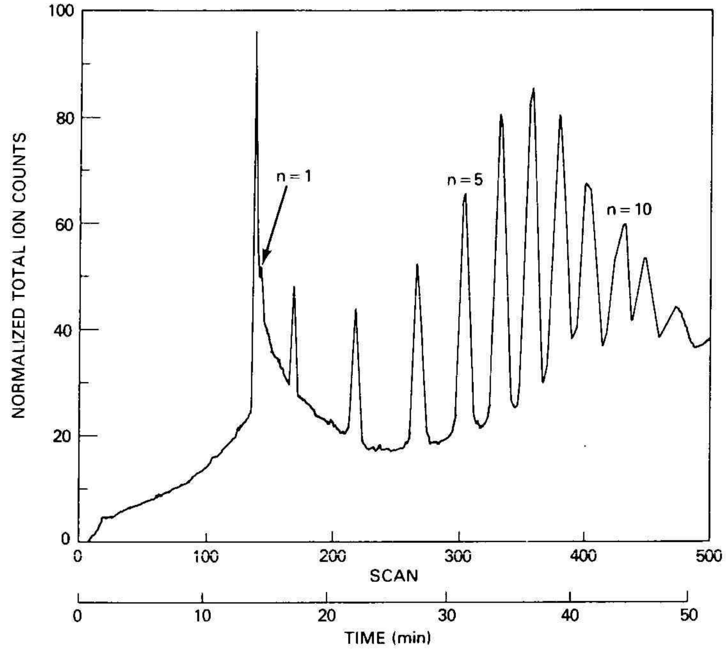


Fig. 8. Total ion chromatogram for the SFC–MS analysis of a polystyrene sample having an average molecular weight of 800. The chromatogram shows the first twelve oligomers which have molecular weights of $104n + 58$. The separation used a 20 m × 0.2 mm I.D. column and isobaric conditions for the first 15 min followed by a 1.2 atm/min increase to 60 atm.

CONCLUSIONS

Excellent sensitivities for a variety of compounds have been demonstrated with the developed capillary column SFC–MS interface. Our initial work has also demonstrated separations comparable to those obtained with HPLC. In principle, capillary SFC should be capable of significantly improved resolution compared with conventional packed column HPLC[16]. The range of samples amenable to SFC approaches that of HPLC and, if substantially increased chromatographic resolution can be obtained, may make SFC the method of choice for many separations. Present limitations upon capillary SFC stem primarily from limited column technology and the non-availability of sufficiently stable or chemically bonded stationary phases.

The development of a reliable SFC–MS interface can be expected to result in the increased application of SFC, particularly as improved and alternative capillary columns become available. This work has demonstrated that the mass spectrometer is a nearly ideal capillary column SFC detector, providing comparable or greater sensitivity than alternative methods in addition to high selectivity. We have also shown that non-volatile high-molecular-weight compounds can be efficiently detected and analyzed using the capillary SFC–MS instrumentation. The detection of such compounds illustrates the successful application of a short capillary restriction for injection of non-volatile molecules into the ionization region. Efforts are being made to simplify the interface by the production of improved restriction devices.

ACKNOWLEDGEMENTS

We thank Dr. W. D. Felix for helpful discussions and A. W. Madsen for assistance in the design and fabrication of the SFC–MS interface.

REFERENCES

1 W. H. McFadden, *J. Chromatogr. Sci.*, 17 (1979) 2–17.
2 P. J. Arpino and G. Guiochon, *Anal. Chem.*, 51 (1979) A682–A701.
3 W. H. McFadden, *J. Chromatogr. Sci.*, 18 (1980) 97–115.
4 N. J. Alcock, C. Eckers, D. E. Games, M. P. L. Games, M. S. Lant, M. A. McDowall, M. Rossiter, R. W. Smith, S. A. Westwood and H.-Y. Wong, *J. Chromatogr.*, 251 (1982) 165–174.
5 M. Dedieu, C. Juin, P. J. Arpino, J. P. Bounine and G. Guiochon, *J. Chromatogr.*, 251 (1982) 203–214.
6 M. N. Myers and J. C. Giddings, *Separ. Sci.*, 1 (1966) 761.
7 S. T. Sie, W. Van Beersum and G. W. Rijnders, *Separ. Sci.*, 1 (1966) 459.
8 R. E. Jentoft and T. H. Gouw, *J. Chromatogr. Sci.*, 8 (1970) 135.
9 E. Klesper, *Angew. Chem. Int. Ed. Engl.*, 17 (1978) 738.
10 T. H. Gouw and R. E. Jentoft, *Advan. Chromatogr.*, 13 (1975) 1–40.
11 L. G. Randall and A. L. Wahrhaftig, *Anal. Chem.*, 50 (1978) 1705–1707.
12 L. G. Randall, *Ph. D. Thesis*, University of Utah, 1979.
13 L. G. Randall and A. L. Wahrhaftig, *Rev. Sci. Instrum.*, 52 (1981) 1283–1295.
14 S. R. Springston and M. Novotny, *Chromatographia*, 14 (1981) 679–684.
15 M. Novotny, S. R. Springston, P. A. Peaden, J. C. Fjeldsted and M. L. Lee, *Anal. Chem.*, 53 (1981) A407–A414.
16 P. A. Peaden, J. C. Fjeldsted, M. L. Lee, S. R. Springton and M. Novotny, *Anal. Chem.*, 54 (1982) 1090–1093.
17 P. A. Peaden, B. W. Wright, M. L. Lee, T. J. Stark, B. A. Newton and P. L. Larson, *Anal. Chem.*, in preparation.
18 K. Grob, G. Grob and K. Grob, Jr., *J. Chromatogr.*, 211 (1981) 243–246.
19 K. Grob and G. Grob, *J. Chromatogr.*, 213 (1981) 211–221.
20 F. J. Van Lenten and L. D. Rothman, *Anal. Chem.*, 48 (1976) 1430–1432.
21 R. A. Yost and C. G. Enke, *J. Amer. Chem. Soc.*, 100 (1978) 2274–2275.
22 M. A. Baldwin and F. W. McLafferty, *Org. Mass Spectrom.*, 7 (1973) 1355–1354.
23 K. H. Schäfer and K. Levsen, *J. Chromatogr.*, 206 (1981) 245–252.
24 P. J. Arpino, P. Krien, S. Vajta and G. Devant, *J. Chromatogr.*, 203 (1981) 117–130.
25 J. D. Henion and T. Wachs, *Anal. Chem.*, 53 (1981) 1963–1965.
26 P. Krien, G. Devant and M. Hardy, *J. Chromatogr.*, 251 (1982) 129–139.

Journal of Chromatography, 247 (1982) 245–254
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,077

DECOMPOSITION OF NITROPYRENE DURING GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF AIR PARTICULATE AND FLY-ASH SAMPLES

J. A. SWEETMAN and F. W. KARASEK*
Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1 (Canada)
and
D. SCHUETZLE
Analytical Sciences Department, Engineering and Research Staff-Research, Ford Motor Company, Dearborn, MI 48121 (U.S.A.)

(Received June 1st, 1982)

SUMMARY

1-Nitropyrene was found to decompose during gas chromatographic–mass spectrometric (GC–MS) analysis. Decomposition was observed both on the column and after the column. The decomposition product was tentatively identified as aminopyrene. Extracts of an air particulate sample and an electrostatically precipitated fly-ash sample from a municipal incinerator were examined for the presence of 1-nitropyrene. The compound was identified in the air particulate extract at the low ppm level, but was not detected in the fly-ash sample. A stable isotope standard was employed to compensate for the lability of the 1-nitropyrene in the GC–MS system to assure positive identification and quantification of the low levels present in air particulates.

INTRODUCTION

There is an increasing awareness that the mutagenicity of airborne particulate matter cannot be attributed solely to the polycyclic aromatic hydrocarbons (PAHs) present. Direct-acting mutagens, that is, compounds which unlike the PAHs are mutagenic for *Salmonella typhimurium* strains without activation by a mammalian metabolic system, have been found in airborne particulates[1], diesel particulate extracts[2,3] and fly-ash[4,5]. The presence of nitroaromatics in airborne particulates has been implicated through the use of bacterial strains of differing sensitivity to nitroaromatics[1]. By fluorescence quenching after thin-layer chromatography, 3-nitrofluoranthene and 6-nitrobenzo[*a*]pyrene have been identified in atmospheric samples[6]. 1-Nitropyrene has been quantified in diesel particulate extracts[2,3,7] and has been found to account for up to 30% of the direct-acting mutagenicity of a diesel exhaust extract[2].

In this study we have examined pooled air particulate sample extracts and pooled municipal incinerator fly-ash extracts for the presence of 1-nitropyrene using gas chromatography mass spectrometry (GC–MS). Nitropyrene was present in the air particulate sample and undetectable in the fly-ash sample. Decomposition of nitropyrene in the GC–MS system made positive identification and quantification difficult at the low levels present in the environmental samples. The use of deuterated (d_9) 1-nitropyrene as an internal standard allowed the positive identification and quantification of 1-nitropyrene.

EXPERIMENTAL

Sample collection and extraction

The air particulate sample was a pooled extract from two urban sampling sites in southern Ontario. The samples were supplied by the Ontario Ministry of the Environment, Air Resources Branch. The particulates were collected on 8 × 10 in. glass-fiber filters using a standard Hi-Vol technique[8]. The filters were extracted overnight with benzene using a Soxhlet extraction apparatus. The dry weight of the combined extracts was 20.2 mg.

The fly-ash sample was a pooled extract from eleven samples of fly-ash from a municipal incinerator in Ontario. The methods of collection and extraction have been described previously[9]. The samples were of electrostatically precipitated fly-ash and were collected on different days. About 20 g of each fly-ash sample were extracted with benzene in a Soxhlet apparatus. The dry weight of the combined extracts was 16.3 mg.

Analysis

A 1-nitropyrene and a deuterated (d_9) 1-nitropyrene standard were received from Ford Motor Company (Dearborn, MI, U.S.A.). 1-Aminopyrene was purchased from Aldrich (Milwaukee, WI, U.S.A.). The high-performance liquid chromatographic (HPLC) separation procedure described by Schuetzle *et al.*[2] was used to fractionate the samples. A 10.7-mg aliquot of the air particulate sample was fractionated and an 8.9-mg aliquot of the fly-ash extract was separated on a semi-preparative scale Porasil 10-μm normal-phase column. A 1-nitropyrene standard was used to determine the time during the gradient program when the fraction should be collected for nitropyrene analysis.

The GC–MS analyses were performed using an HP5992 GC–MS-calculator system equipped with an x–y plotter and floppy disk. Packed column runs were carried out on a 6-ft. column packed with high-performance Aue packing[10]. A membrane separator interfaced the packed column to the MS system. Analysis by capillary GC was carried out on a 25-m fused silica OV-101 column with splitless injection. An HP59916A glass capillary effluent splitter interfaced the capillary column to the MS systen. The mass spectrometer was tuned using the manufacturer-supplied program AUTOTUNE.

RESULTS AND DISCUSSION

The electron-impact fragmentation pattern of 1-nitropyrene as reported by Schuetzle *et al.*[2] is given in Table I. These abundances are in good agreement with the

TABLE I

RELATIVE ABUNDANCES (%) OF IONS IN 1-NITROPYRENE AND 1-AMINOPYRENE

m/z	*1-Nitropyrene*					*1-Aminopyrene*
	Schuetzle et al.[2]	*480 ng injected*	*130 ng injected*	*35 ng injected*	*15 ng injected*	
247	100	90,90,93	88,82	59,58	30,31	–
201	100	100,100,100	100,100	72,70	39,41	2,2
200	55	74,75,74	77,72	62,55	39,34	3,3
217	40	49,61,50	60,71	100,100	100,100	100,100
189	40	56,56,54	59,60	62,65	61,50	35,39
216	–	6,7,5	9,10	22,18	24,25	21,20
108.5	–	3, $<$ 5,2	5,6	20,15	24,22	23,26
94	–	5,7,5	8,10	21,18	33,34	31,34

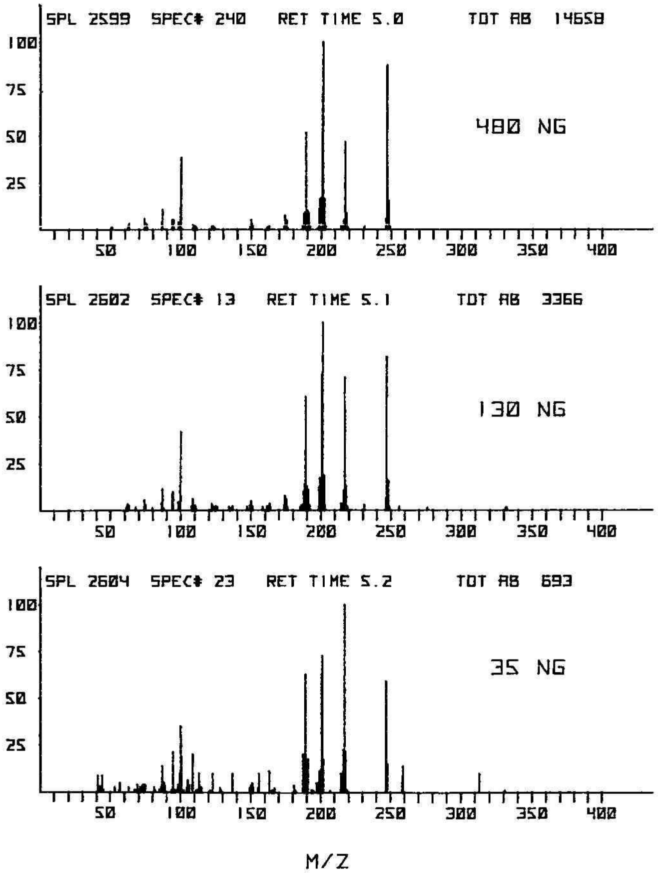


Fig. 1. Mass spectra for three amounts of 1-nitropyrene. The small peaks at *m/z* = 259 and 313 of the 35-ng injection are due to the background.

spectrum identified by Yu and Hites[11] as nitropyrene or nitrofluoranthene in a diesel particulate extract sample. Our spectra of high concentrations of 1-nitropyrene agree well with these published spectra. However, it was found that when smaller amounts of 1-nitropyrene were injected these relative abundances were not observed.

Fig. 1 shows the spectra obtained for 1-nitropyrene for three levels of compound injected. The 1-nitropyrene was introduced by on-column injection on a packed column. The spectra were taken at the peak maxima, with the lowest valley between peaks used for background subtraction. The ions at *m/z* = 217, 216, 108.5 and 94 increased in relative abundance as lower amounts of 1-nitropyrene were injected. Fig. 2 shows that these ions are all present in the spectrum of 1-aminopyrene. As can be seen from Table I, for the 15–35-ng 1-nitropyrene injections, the relative ion abundances of *m/z* 217/216, 217/108.5 and 217/94 all approach the values found in 1-aminopyrene spectra. This suggests that the spectra in Fig. 1 are combined spectra of 1-nitro- and 1-aminopyrene with the 1-aminopyrene to 1-nitropyrene ratio increasing with decreasing amounts of 1-nitropyrene injected. If, as suggested by the data in Table I, the presence of ions at *m/z* = 216, 108.5 and 94 is due to 1-aminopyrene, then the *m/z* = 108.5 peak is due to the doubly charged molecular ion of 1-aminopyrene.

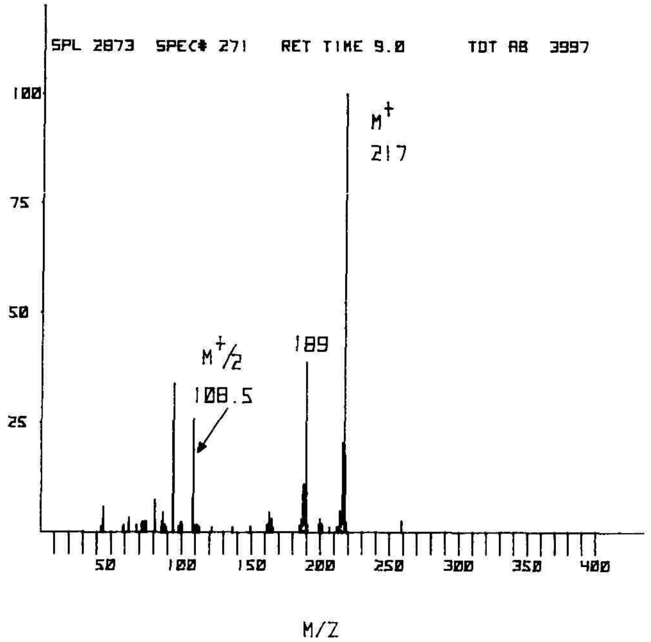


Fig. 2. Mass spectrum of 1-aminopyrene.

Possible explanations for the presence of 1-aminopyrene are that it is an impurity in the standard 1-nitropyrene or that the nitropyrene decomposes to 1-aminopyrene somewhere in the GC–MS system. Fig. 3 shows a selected-ion monitoring (SIM) trace of a co-injection of 1-nitropyrene and 1-aminopyrene. As the two compounds are separated on the packed column employed, a 1-aminopyrene impurity in

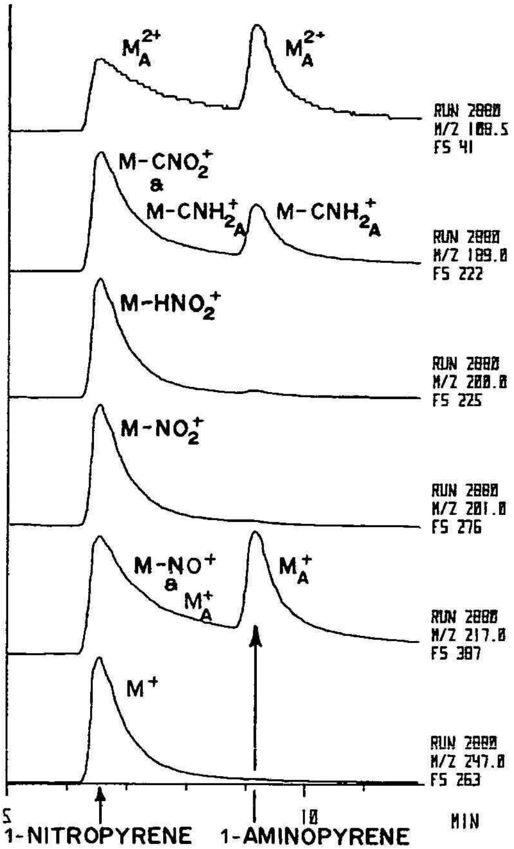


Fig. 3. SIM data for a co-injection of 1-nitropyrene (101 ng) and 1-aminopyrene (97 ng). Aue 6-ft. packed column. Conditions: injection port temperature, 250°C; initial column temperature, 220°C for 3 min, then programmed at 4°C/min to 250°C. Subscript A indicates ion of 1-aminopyrene. FS = full-scale value.

the standard could not explain the 1-aminopyrene present in the spectra in Fig. 1. Decomposition in the GC–MS system would explain the observed spectra. For decomposition on the column, the longer retention time of the compound as it decomposed to 1-aminopyrene would produce a tailing peak. It can be seen in Fig. 3 that the ions characteristic of 1-aminopyrene, *i.e.*, m/z = 217, 189 and 108.5, are indeed tailing peaks. The ions at m/z = 247, 201 and 200, which are present only in 1-nitropyrene,-produce more nearly symmetrical peaks.

In addition to on-column decomposition of 1-nitropyrene to 1-aminopyrene, it would appear that decomposition is occurring at the GC–MS interface or in the mass spectrometer source. The m/z = 247 peak is due entirely to the 1-nitropyrene, so the peak maximum of this peak gives the retention time of nitropyrene on the column. In Fig. 3 the high relative abundance of the m/z = 217 ion in comparison with the literature spectra of 1-nitropyrene indicates some 1-aminopyrene to be present. As the peak maximum of the m/z = 217 ion is coincident with that of m/z = 247, this indicates that the compound traveled through the column as nitropyrene and decomposed after the column at the interface or in the MS source. The appearance of a peak at m/z = 108.5 with the same retention times maximum also suggests decomposition after the column, as this peak is the doubly charged molecular ion peak seen in the spectrum of 1-aminopyrene. Suggested identities of the fragment ions are given in Fig. 3.

The same change in spectral pattern with concentration occurred using a fused silica capillary column (OV-101) with splitless injection. Fig. 4 shows the m/z = 247 $(M)^+$ and m/z = 217 ions for a SIM run with 18 ng of 1-nitropyrene standard. Note that no molecular ion peak is present, suggesting that the 1-nitropyrene is entirely decomposed.

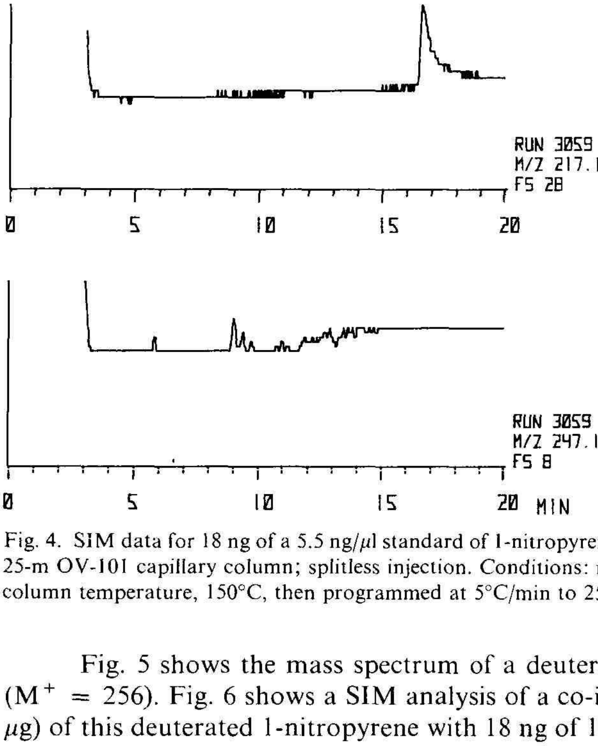


Fig. 4. SIM data for 18 ng of a 5.5 ng/μl standard of 1-nitropyrene. m/z = 247 is the molecular ion, $(M)^+$. 25-m OV-101 capillary column; splitless injection. Conditions: injection port temperature, 250°C; initial column temperature, 150°C, then programmed at 5°C/min to 250°C.

Fig. 5 shows the mass spectrum of a deuterated (d_9) 1-nitropyrene standard (M^+ = 256). Fig. 6 shows a SIM analysis of a co-injection of a large amount (*ca.* 1 μg) of this deuterated 1-nitropyrene with 18 ng of 1-nitropyrene standard. A distinct $(M)^+$ peak for 1-nitropyrene is now present at m/z = 247 and the abundances of both m/z = 247 and 217 are increased (compare with Fig. 4). Fig. 7 shows a SIM run of 1-nitropyrene-d_9 monitoring the $(M)^+$, $(M - NO)^+$ and $(M - CNO_2)^+$ peaks for both 1-nitropyrene and 1-nitropyrene-d_9. The very low full-scale value for the m/z = 247 ion shows that there is no interference of the 1-nitropyrene-d_9 with the molecular ion of 1-nitropyrene, that is, the m/z = 247 abundance in Fig. 6 is due entirely to the 1-nitropyrene. The m/z = 217 abundance, however, is in part from the deuterated standard.

By injecting a large amount of deuterated standard, the fraction decomposing is small. As the active sites causing decomposition do not distinguish deuterated from non-deuterated, the fraction of 1-nitropyrene decomposing is also small and the m/z = 247 peak reflects the actual concentration. The estimated minimum detectable amount of 1-nitropyrene, that is, the minimum amount of 1-nitropyrene which when

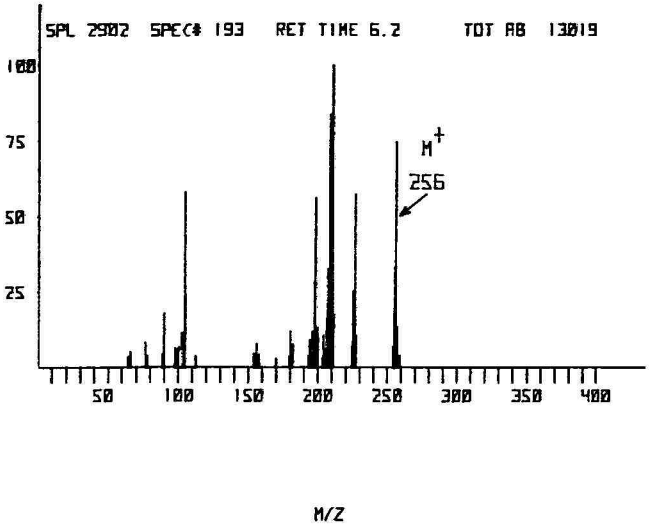


Fig. 5. Mass spectrum of 1-nitropyrene-d_9.

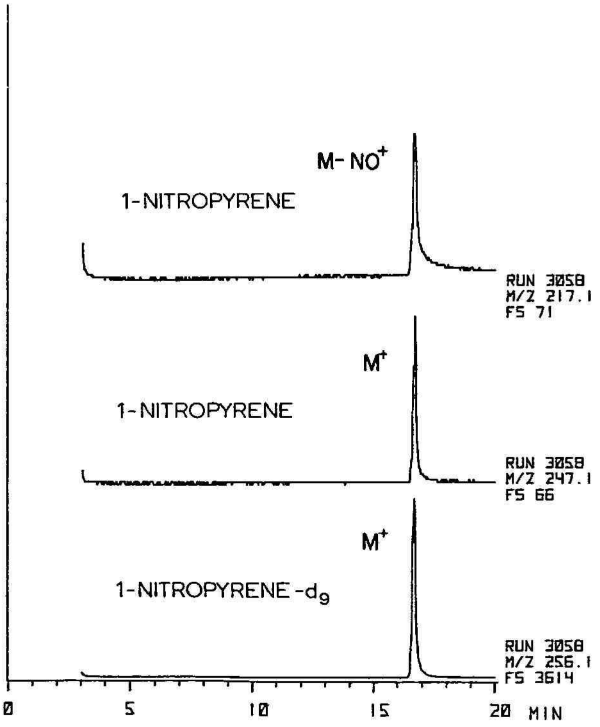


Fig. 6. SIM data for 18 ng of 1-nitropyrene standard co-injected with *ca.* 1 μg of 1-nitropyrene-d_9. Column and conditions as in Fig. 4.

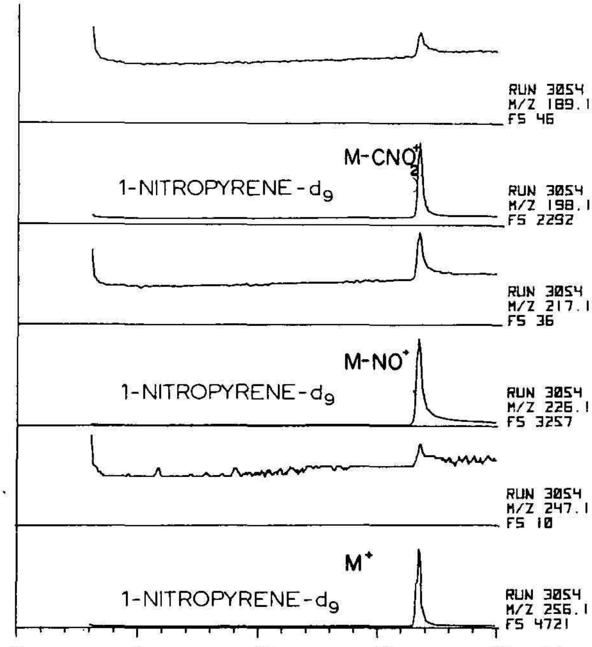


Fig. 7. SIM data for *ca.* 1 μg of 1-nitropyrene-d_9, monitoring the $(M)^+$, $(M - NO)^+$ and $(M - CNO_2)^+$ ions for 1-nitropyrene-d_9 and 1-nitropyrene. Column and conditions as in Fig. 4. Note the insignificant $m/z = 247$ abundance.

co-injected with 1 μg of 1-nitropyrene-d_9 would give a discernible $m/z = 247$ peak, is 3 ng.

The HPLC fraction of the air particulate sample in which 1-nitropyrene would be eluted was examined by GC–MS–SIM. Fig. 8 shows the air particulate fraction injected alone (bottom two ion traces) and co-injected with 1-nitropyrene-d_9. The appearance of a peak at $m/z = 247$ which coincides with a peak at $m/z = 217$ and matches the retention time of the $(M)^+$ peak of $m/z = 256$ for 1-nitropyrene-d_9 identifies this peak as 1-nitropyrene. One or more of the three earlier peaks seen on the $m/z = 217$ ion trace could be isomers of nitropyrene or nitrofluoranthene with no discernible $(M)^+$ peak.

The average response (area of $m/z = 256$ per ng) for four injections of 1.1 μg of 1-nitropyrene-d_9 was 435 area counts. The standard deviation of the four numbers was 60. This relatively large standard deviation may be attributable to the difficulties with reproducibility in the splitless injection mode and to variable adsorption effects. The spectral pattern as evidenced by the ratio of $m/z = 256/226$ remained constant.

For the air particulate sample, the molecular ion was used for quantification as there is no interference from the deuterated standard. Using the co-injected deuterated standard as an internal standard to correct for non-uniform adsorption and injection, corrected response (area of $m/z = 247$ per ng) values for duplicate injections of the 5.5 ng/μl of 1-nitropyrene standard were 382 and 399. By comparing Figs. 6 and 8, it can be seen that the 5.5 ng/μl standard closely corresponds to the amount of 1-nitropyrene in the air particulate sample injections. Therefore, the average value

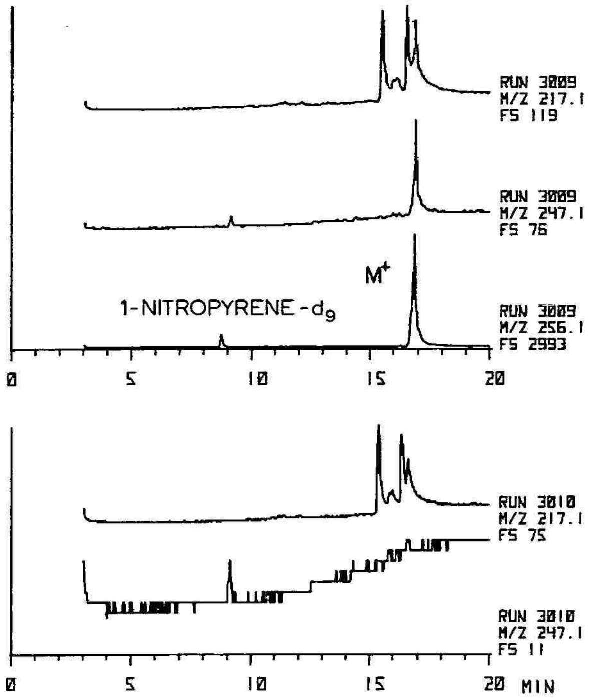


Fig. 8. SIM data for an air particulate extract sample, injected alone (lower two traces) and co-injected with *ca.* 1 μg of 1-nitropyrene-d_9 (upper three traces). Column and conditions as in Fig. 4. Each injection was 3% of the air particulate HPLC fraction.

of 390 (area of $m/z = 247$ per ng of nitropyrene) was used to calculate the concentration of 1-nitropyrene in the air particulate sample injections. Accounting for dilution volumes and the total air particulate extract weight, the 1-nitropyrene concentration in the extract is 60 and 68 ppm (w/w) for the duplicate quantifications.

For the HPLC fraction of the fly-ash extract in which 1-nitropyrene would appear, if present, no discernible peaks at $m/z = 247$ or 217 appear at the retention time for 1-nitropyrene. Co-injection of the 1-nitropyrene-d_9 standard produced only a small interference peak at $m/z = 217$. Owing to a high background from the fly-ash for $m/z = 247$, the detection limit for 1-nitropyrene in this sample is about 20 ppm (w/w) of extract.

Although 1-nitropyrene was not detected in this precipitated fly-ash from a municipal incinerator, the presence of 1-nitropyrene in the exhaust gases from the incinerator cannot be ruled out. Fisher *et al.*[4] have reported that ash samples collected from the hoppers of an electrostatic precipitator in a coal-fired power plant were not mutagenic, whereas fly-ash collected from the smokestack of the power plant were found to be mutagenic.

The decomposition of 1-nitropyrene in the GC–MS system makes positive identification and quantification difficult. The use of a deuterated internal standard allowed the quantification of 1-nitropyrene in an air particulate sample.

ACKNOWLEDGEMENT

This work was supported under a research agreement with the Ford Motor Company of Canada, Limited.

REFERENCES

1 C. Y. Wang, M.-S. Lee, C. M. King and P. O. Warner, *Chemosphere*,9 (1980) 83.
2 D. Schuetzle, F. S.-C. Lee anc T. J. Prater, *Int. J. Environ. Anal. Chem.*, 9 (1981) 93.
3 T. L. Gibson, A. I. Ricci and R. L. Williams, *GMR-3478, ENV No. 95*, Research Publication, General Motors Research Laboratories, Warren, MI, 1980.
4 G. L. Fisher, C. E. Chrisp and O. G. Raabe, *Science*, 204 (1979) 879.
5 C. E. Chrisp, G. L. Fisher and J. E. Lammer, *Science*, 199 (1978) 73.
6 J. Jaeger, *J. Chromatogr.*, 152 (1978) 575.
7 D. Schuetzle, T. L. Riley, T. J. Prater, T. M. Harvey and D. F. Hunt, *Anal. Chem.*, 54 (1982) 265.
8 G. A. Jutze and K. E. Foster, *J. Air Pollut. Control Assoc.*, 17 (1967) 17.
9 G. A. Eiceman, R. E. Clement and F. W. Karasek, *Anal. Chem.*, 53 (1981) 955.
10 H. H. Hill, Jr., K. W. Chan and F. W. Karasek, *J. Chromatogr.*, 131 (1977) 245.
11 M.-L. Yu and R. A. Hites, *Anal. Chem.*, 53 (1981) 951.

Journal of Chromatography, 247 (1982) 255–262
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,067

IDENTIFICATION AND DETERMINATION OF SOME UREA, CARBAMATE AND THIOCARBAMATE DERIVATIVES IN AIR

L. G. ALEXANDROVA*
Kiev Research Institute of Labour, Hygiene and Occupational Diseases, Kiev (U.S.S.R.)
and
M. A. KLISENKO
All-Union Research Institute of Hygiene and Toxicology of Pesticides, Polymers and Plastics, Kiev (U.S.S.R.)

(Received March 15th, 1982)

SUMMARY

The use of UV spectrophotometry, thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) for the identification and determination of some carbamate, thiocarbamate acid and urea derivatives is described. For the separation of carbamate and thiocarbamate pesticides TLC and GLC are applied. Methods for obtaining airborne samples are described and the results of the determination of some pesticides by different methods are presented.

INTRODUCTION

The increasing use of herbicides and pesticides has led to environmental contamination problems, including air. Among the many chemical combinations used as pesticides, urea, carbamate and thiocarbamate derivatives are of great importance. This paper describes methods for the identification and determination of such compounds in air.

In accordance with requirements[1] for methods for air analysis in working zones, the duration of taking air samples should not exceed 30 min when determining pesticides at the level of half of the maximum allowable concentration (MAC). Proceeding from these requirements, the lower limit of detection of the substance in 1 m^3 of air should not exceed half of the MAC.

Table I lists the MACs of carbamate, thiocarbamate and urea derivatives[1] and the maximum possible concentrations of their vapours in air calculated by us according to their saturated vapour pressures[2,3] and also obtained experimentally are given. As shown in Table I, the MAC of pesticides in air in working zones fluctuates within the range 0.2–5 mg m^{-3} and the maximum possible concentrations of their vapours in air are 0.12–54.2 mg m^{-3} for carbamates, 0.03–0.3 mg m^{-3} for urea and 23–357 mg m^{-3} for thiocarbamates. Hence maximum possible concentrations of pesticide vapours exceeding the MAC by many times occur for some carbamate derivatives and all

TABLE I

MAC AND MAXIMUM POSSIBLE VAPOUR CONCENTRATION OF SOME PESTICIDES IN THE WORKING ZONE AIR

Systematic name	*Pesticide name*		*MAC ($mg\ m^{-3}$)*	*Saturated vapour pressure (mmHg)*	*Max. possible concentration ($mg\ m^{-3}$)*
	ISO	*U.S.S.R.*			
3-Methoxycarbonylaminophenyl-N-(3-methylphenyl)carbamate	Phenmedipham	Betanal	0.5	$0.14 \cdot 10^{-4}$	0.2(20°C)
4-Chlorbutyne-2-yl-N-*m*-chlorophenylcarbamate	Barban	Carbyne	0.5	$5.02 \cdot 10^{-4}$	7(25°C)
Isopropyl-N-phenylcarbamate	Propham	IFC	2.0		6.4(20°C)
Isopropyl-N-(3-chlorophenylcarbamate)	Chlorpropham	Chlor-IFC	2.0	10^{-5}–10^{-6}	0.1 0.01(25°C)
1-Naphthyl-N-methylcarbamate	Carbaryl	Sevin	1.0	$5 \cdot 10^{-3}$	54.2(26°C)
5,6-Dimethyl-2-dimethylamino-4-pyrimidinyldimethylcarbamate	Pyrimicarb	Pyrimor	0.2	$30 \cdot 10^{-6}$	0.37(30°C)
S-Propyldipropylthiocarbamate	Vernolat	Vernam	5.0	$10.9 \cdot 10^{-3}$	119.4(25°C)
S-Ethylcyclohexylthiocarbamate	Cycloate	Roneet	1.0	$2 \cdot 10^{-3}$	23.4(25°C) 93(20°C)
S-Ethyl-N,N-diisobutylthiocarbamate	Butylate	Sutan	–	$13.0 \cdot 10^{-3}$	152(25°C)
S-Propyl-N-ethyl-N-butylthiocarbamate	Pebulate	Tillam	1.0	0.01	109.4(25°C)
S-Ethyl-N,N-di-*n*-propylthiocarbamate	EPTC	Eptam	2.0	$35 \cdot 10^{-3}$	357(25°C)
S-Ethyl-1-hexamethyleniminothiocarbamate	Ordram	Yalan	0.5	0.005	56(25°C)
N-3,4-Dichlorphenyl-N,N-dimethylurea	Diuron	Diuron	5.0	$0.31 \cdot 10^{-5}$	0.03(50°C)
1,3-Bis-(2,2,2-trichloro-1-oxyethyl)urea		DCM	5.0		0.3(20°C)

thiocarbamate derivatives listed. This determines the method of taking air samples for pesticide analysis, depending on the state of the substance involved.

In the literature, conditions for the chromatographic separation of numerous pesticides, including phenylurea, carbamate and some thiocarbamate derivatives, from vegetables, soils and animal tissues have been described. The methods used included thin-layer chromatography (TLC) and gas–liquid chromatography (GLC) with flame-photometric, electrolytic conductivity and nitrogen (N-FID) detectors[3–7]. The application of high-performance liquid chromatography (HPLC) for the determination of carbamate insecticide[8] has also been described. There is little information on methods for the determination of urea, carbamate and thiocarbamate derivatives in air[9–11].

We have investigated the possibility of identifying and determining some urea, carbamate and thiocarbamate derivatives in air by means of UV spectrophotometry, TLC and GLC.

EXPERIMENTAL AND RESULTS

UV spectrophotometry

UV absorption spectra of chemically pure substances were measured at room temperature (20 ± 3°C) using SF-4a and SF-16 spectrophotometers in quartz cuvettes (l = 1 cm) with 96% ethanol and *n*-hexane as solvents.

Table II gives wavelengths of maximum absorption and molar absorptivities.

TABLE II

ε_{max} AND λ_{max} OF ABSORPTION OF UREA, CARBAMATE AND THIOCARBAMATE DERIVATIVES

Type	*Pesticide*	*Empirical formula*	λ_{max}	ε_{max} ($l\ mol^{-1}\ cm^{-1}$)	*Solvent*
Thiocarbamates	Diptal	$C_{10}H_{16}ONClS$	249	$0.31 \cdot 10^4$	*n*-Hexane
			254–255	$0.56 \cdot 10^4$	Ethanol
	Roneet	$C_{11}H_{21}ONS$	233	$1.54 \cdot 10^3$	*n*-Hexane
			272	$0.33 \cdot 10^3$	
			232	$0.88 \cdot 10^3$	Ethanol
			272	$0.14 \cdot 10^3$	
	Eptam	$C_9H_{19}ONS$	255	$0.67 \cdot 10^3$	*n*-Hexane
			255	$0.14 \cdot 10^3$	Ethanol
	Yalan	$C_9H_{17}ONS$	280–281	$0.63 \cdot 10^2$	*n*-Hexane
			279–280	$1.5 \cdot 10^2$	Ethanol
Arylcarbamates	Asulam	$C_8H_{10}O_4N_2S$	270	$1.6 \cdot 10^4$	Ethanol
	Betanal	$C_{16}H_{16}O_4N_2$	238	$3.72 \cdot 10^4$	Ethanol
			262.5	$0.3 \cdot 10^4$	Ethanol
			265	$0.35 \cdot 10^4$	*n*-Hexane
			278	$0.35 \cdot 10^4$	*n*-Hexane
	Carbyne	$C_{11}H_9O_2NCl_2$	277–278	$1.8 \cdot 10^3$	*n*-Hexane
			285–286	$1.6 \cdot 10^3$	Ethanol
	IFC	$C_{10}H_{13}O_2N$	237		Ethanol
			275	$0.85 \cdot 10^3$	*n*-Hexane
			282–286		
	Chlor-IFC	$C_{10}H_{12}O_2NCl$	240–241	$2.65 \cdot 10^3$	Ethanol
			273	$0.48 \cdot 10^3$	Ethanol
			274	$1.39 \cdot 10^3$	*n*-Hexane
			286		*n*-Hexane
	Aniline	C_6H_7N	230, 280		*n*-Hexane
	m-Chloroaniline	C_6H_6NCl	239.5, 285–286		*n*-Hexane
Phenylurea derivatives	Dosanex	$C_{10}H_{13}O_2N_2Cl$	247	$1.05 \cdot 10^4$	Ethanol
			247	$0.2 \cdot 10^4$	*n*-Hexane
	Diuron	$C_9H_{10}ON_2Cl_2$	246	$1.6 \cdot 10^3$	Ethanol
	3,4-Dichloroaniline	$C_6H_5NCl_2$	244–245		Ethanol

TLC analysis

Aluminium oxide (activity II), silica gel KCK and Silufol TLC plates were used, with *n*-hexane, *n*-heptane, chloroform, benzene, ethanol, ethyl acetate, carbon tetrachloride, diethyl ether, light petroleum, acetone and their mixtures as mobile phases.

For the detection of phenylcarbamates and phenylureas, an azo reaction was used. Diazotization was carried out with sodium nitrite solution after preliminary thermal degradation of the pesticide molecules, then the diazo compound obtained was combined with α-naphthol or α-naphthylamine. Coloured reaction products were obtained with 4-aminoantipyrine in the presence of ammonium persulphate [$(NH_4)_2S_2O_8$] as an oxidant (betanal), or with *p*-dimethylaminobenzaldehyde (asulam, betanal). The sensitivity of determination was 1–10 μg.

Thiocarbamate pesticides were subjected to reaction with bromophenol blue reagent or with *o*-toluidine after N-chlorination (eptam, tillam, yalan). The sensitivity was 0.25–2 μg. For detection of roneet, Dragendorf reagent or sulphuric acid were also used.

Table III gives the optimum conditions for the determination of some combinations.

TABLE III

LIMITS OF DETECTION AND R_F VALUES OF CARBAMATE AND THIOCARBAMATE DERIVATIVES IN THIN LAYERS OF SORBENT

Pesticide	*Sorbent*	*Mobile phase**	*R_F value*	*Minimum determinable amount (μg)*
Asulam	Aluminium oxide	E–W (2:1)	0.55	3
Betanal	Aluminium oxide	C	0.4	3
Benleit	Silufol	CCl_4–E (9:1)	0.6	1
Carbyne	Aluminium oxide	H–C (1:3)	0.5	5
IFC	Aluminium oxide	H–C (1:3)	0.76	10
Chlor-IFC	Aluminium oxide	H–C (1:3)	0.8	10
Aniline	Aluminium oxide	H–C (1:3)	0.62	
m-Chloroaniline	Aluminium oxide	H–C (1:3)	0.71	
Yalan	Silufol	H–D (4:1)	0.54	1
Roneet	Silufol	H–D (4:1)	0.73	2
Tillam	Silufol	H–D (4:1)	0.80	2
Vernam	Silufol	H–D (4:1)	0.82	0.25
Sutan	Silufol	H–D (4:1)	0.87	1
Yalan	Silufol	B–H (9:1)	0.6	1
Roneet	Aluminium oxide	B–H (3:2 or 4:1)	0.55	10

* B = benzene; C = chloroform; CCl_4 = carbon tetrachloride; D = diethyl ether; E = ethanol; H = *n*-hexane; W = water.

GLC analysis

A Tswet-106 gas chromatograph with a thermoionic detector (TID) was used under both isothermal conditions and with temperature programming. The lengths of the glass columns were 1 and 2 m, with I.D. 3 mm.

The stationary phases SE-30 (3 and 5%), XE-60 (5%), DC-550 (5%) and OV-17 (2 and 5%) on Chromaton N AW DMCS and Chromaton N AW (0.16–0.24 mm) were investigated at different temperatures.

The following optimum conditions for the separation of six thiocarbamate pesticides were found: column temperature, 140–170°C; nitrogen flow-rate, 20 ml min^{-1}; compressed air flow-rate, 200–400 ml min^{-1}; hydrogen flow-rate 15 ml min^{-1}; amplification, $5 \cdot 10^{-12}$–$100 \cdot 10^{-12}$ A.

Table IV gives results for the separation of thiocarbamate derivatives using a TID and 5% DC-550 on Chromaton N AW DMCS and a 5% SE-30–5% XE-60 (1:1) mixed phase.

TABLE IV

SEPARATION OF SOME THIOCARBAMATE DERIVATIVES BY CHROMATOGRAPHY

Pesticide	*5% DC-550*		*5% SE-30 + 5% XE-60 (1:1)*	
	Retention time (sec)	*Separation factor*	*Retention time (sec)*	*Separation factor*
Eptam	270		285	
		1.1		1.5
Sutan	348		348	
		0.4		0.6
Vernam	405		375	
		0.3		2.5
Tillam	437		690	
		1.1		1.0
Yalan	712		750	
		1.3		0.7
Roneet	954		910	

Air sampling

When applied in agriculture, the derivatives of urea (DCM, diuron and others) and of some carbamates such as betanal, chlor-IFC, carbyne and pirimor occur mainly as aerosols in air in the working zone and thiocarbamates occur as aerosols and vapours simultaneously. Therefore, when studying conditions for air sampling (absorption media, rate of sampling, duration of aspiration), the pesticide volatility, the form of its application (concentrate, emulsion, granulated preparation, etc.), and the method of treatment of agricultural samples must be taken into account. Hence, when sampling aerosols, the filters investigated were AFA-KhA (filtration material: uniform distribution of ultrathin acetylcellulose fibres) and AFA-V (Perchlor vinyl fibre) ash-free filter-papers.

The rate of air aspiration was up to 15 l min^{-1}. For sampling vapours, silica gel KSK, ASK, glass-wool, acetone, *n*-hexane and ethanol in cooling traps were used as absorbents. The sampling rate was 0.2–1 l min^{-1}.

The absorption capacity of the absorption media was tested in model experiments under laboratory conditions; using an experimental aerosol an aspiration rate was selected and the air volume necessary for analysis was determined. When taking samples of airborne volatile pesticides such as vernam and eptam, combined absorbents (successive filters containing liquid absorbents or silica gel) were used.

DISCUSSION

UV spectrophotometry has been applied to the determination of microamounts of pesticides in waters, soils, vegetables and some food products. The use of UV spectrophotometry permits the identification of the products of pesticide transformations in living organisms and the environment and provides information on the processes of pesticide photolysis in the biosphere, because the presence of maxima in the UV spectra indicates the capacity of the molecules to be excited and to undergo specific reactions when UV irradiated.

From Table II it can be seen that maxima at 233–255 and 272–280 nm are a peculiarity of the absorption spectra of thiocarbamate derivatives. The absorption spectrum of tetramethylthiourea, with λ_{max} = 255.5 nm (ε = 17,000 l mol^{-1} cm^{-1}), is the result of the effect of conjugation between the thiocarbonyl group and free pair of electrons on the adjacent nitrogen atoms. The presence of an intense maximum at 246–247 nm (ε = 1600–10,500 l mol^{-1} cm^{-1}) is an absorption peculiarity of urea derivatives. The UV spectra of ether arylcarbamate had an intense maximum near 236–241 nm. These maxima are conditioned mainly by the change in the electrons in the aromatic ring. The limit of detection of the pesticides by spectrophotometry is 0.5–5 mg m^{-3}, as shown in Table II. The sensitivity of the spectrophotometric method thus satisfies the requirements for methods for the analysis of air in working zones, but its practical application is often limited because of the difficulty of the separate determination of the components of some airborne combinations, the maxima of which coincide or are close together.

For establishing the possibility of separating pesticides with similar structures, TLC was examined. The dependence of the chromatographic parameters on the chemical structure of molecular combinations studied and on the sorbent and the mobile phase was investigated. In TLC using *n*-hexane with thin layers of aluminium oxide and silica gel KSK the mobilities of asulam, betanal, benleite, carbyne, IFC, chlor-IFC, yalan, roneet, tillam, vernam and sutan are zero or nearly zero. In chromatography with mobile phases containing *n*-hexane, the R_F value increases as a function of the extent of blocking of the active centres of the adsorbent by the components of the mixture. As chloroform and benzene have specific interactions with the adsorbent, competing with interactions of the adsorbed compounds, the R_F values of pesticides are higher in chloroform and benzene than in *n*-hexane. The R_F values increase considerably when diethyl ether is added to the solvent system, owing to a strong specific interaction between the ether and the adsorbent. The optimum conditions for separating some arylcarbamate derivatives (carbyne, IFC, Chlor-IFC and their aniline and *m*-chloroaniline metabolites) and thiocarbamate derivatives (roneet, tillam, sutan and yalan) were determined. Asulam, betanal and benleite have R_F values of zero or nearly zero under the selected conditions. The detection limit is 0.1 mg m^{-3} (refs. 12–16).

Thiocarbamate derivatives with similar structures, *e.g.*, vernam, sutan and tilam, cannot always be separated and identified reliably. GLC was used for this purpose. The optimum conditions for separating six thiocarbamates were obtained by using a TID with 5% DC-550, 5% XE-60 and mixed 5% SE-30–5% XE-60 (1:1) as the stationary phase. From the data in Table IV, it can be seen that the best separations of sutan and vernam and of vernam and tillam are achieved on the mixed

TABLE V

CONDITIONS OF AIR SAMPLING FOR SOME PESTICIDES

Pesticide	*Aggregate state*	*Adsorbent*	*Sampling rate ($l\ min^{-1}$)*	*Investigated air volume (l)*
Benleit	Aerosol	AFA-KhA	5	200
Carbyne	Aerosol	AFA-KhA	5	20
IFC	Aerosol	AFA-KhA	5	40
Chlor-IFC	Aerosol	AFA-KhA	5	40
DCM	Aerosol + vapour	Ash-free filter paper	5	20
		Acetone	0.3–0.5	10
Vernam	Aerosol + vapour	AFA-KhA + liquid absorber (acetone)	1	1
Roneet	Aerosol + vapour	AFA-KhA	2	10
Tillam	Vapour	Silica gel (or acetone)	0.3–0.4	10
	Aerosol	AFA-KhA	3–5	10
Eptam	Aerosol + vapour	AFA-KhA + liquid absorber (*n*-hexane)	0.4	10
	Aerosol	AFA-KhA	3–5	10
Yalan	Aerosol	Glass-wool; AFA-KhA	5	10

column, and of tillam and yalan and of yalan and roneet on DC-550. The limits of detection of thiocarbamate pesticides in air are 0.02–0.2 mg m^{-3}.

Studies of the air sampling conditions showed that pesticides that occur as aerosols are satisfactorily absorbed by the AFA-KhA and AFA-V ash-free filter-papers. The AFA-KhA filter can be used for vapour sampling when the pesticide concentration is not higher than 5 mg m^{-3}. The results showed that when sampling air containing high-volatility pesticides (*e.g.*, vernam and eptam), two or three AFA-KhA filters have to be used when the concentration is higher than 50 mg m^{-3}. When the concentrations are higher than 100 mg m^{-3} a control absorbing apparatus with a liquid absorber must be connected in series with the filter holder. Table V lists the conditions of air sampling for some pesticides.

Table VI gives the results of pesticide detection by different methods.

TABLE VI

RESULTS OF DETERMINATION OF SOME PESTICIDES BY DIFFERENT METHODS

Initial concentration: 2 MAC; number of determinations is 6–10; all values are within a 95% confidence limit.

Pesticide	*Recovery (%)*		*Pesticide*	*Recovery (%)*	
	UV spectrophotometry	*TLC*		*TLC*	*GLC*
Betanal	91 ± 5.5	90 ± 5	Vernam	89 ± 10.8	90 ± 2.5
Carbyne	97.2 ± 3.5	94 ± 11	Sutan	70.2 ± 5.5	84.5 ± 4.8
IFC	89.8 ± 3.9	89 ± 13.9	Roneet	87 ± 5	88.5 ± 3.4
Chlor-IFC	94.5 ± 4.0	90 ± 12.5	DCM	88 ± 11.5	92 ± 5.6

In conclusion, it appears that the individual pesticides in air can be detected by UV spectrophotometry with high sensitivity. TLC and GLC are effective in separating mixtures, and the combination of these methods makes it possible for the investigated compounds to be identified.

REFERENCES

1 *GOST 12.1.005-76, Vozdukh Rabochei Zony, Obschiye Sanitarno-Gigiyenicheskiye Trebovaniya*, Publishing of Standards, Moscow, 1976.
2 I. Sunshine (Editor), *Handbook of Analytical Toxicology*, Chemical Rubber Publishing Co., Cleveland, OH, 1969, 1081 pp.
3 G. Zweig (Editor), *Analytical Methods for Pesticides and Plant Growth Regulators and Food Additives, Vol. V, Thin-Layer and Liquid Chromatography of Pesticides of International Importance*, Academic Press, New York, London, 1973, 729 pp.
4 G. Zweig (Editor), *Analytical Methods for Pesticides and Plant Growth Regulators, Vol. VI, Gas Chromatographic Analysis*, Academic Press, New York, London, 1972, 550 pp.
5 H. Maier Bode and M. Riedmann, *Residue Rev.*, 54 (1975) 113–181.
6 R. C. Hall and D. E. Harris, *J. Chromatogr.*, 169 (1979) 245–260.
7 T. Kömives, V. A. Aprókovács and A. F. Márton, *J. Chromatogr.*, 175 (1979) 222–223.
8 R. T. Krause, *J. Chromatogr.*, 185 (1979) 615–624.
9 B. Compton, *Prog. Anal. Chem.*, 5 (1973) 133–152.
10 J. N. Seiber, J. E. Woodrow, T. M. Shafik and H. F. Enos, in R. Hague and V. H. Freed (Editors), *Environmental Dynamics of Pesticides*, Plenum Press, New York, London, 1975, pp. 17–43.
11 J. Sherma and T. M. Shafik, *Arch. Environ. Contam. Toxicol.*, 3 (1975) 55–71.
12 L. G. Alexandrova, *Khim. Sel'sk. Khoz.*, No. 9 (1975) 59–60.
13 N. G. Stepanchenko, *Gig. Sanit.*, No. 11 (1976) 82–84.
14 T. A. Lebedeva and G. A. Khokholykova, *Gig. Sanit.*, No. 5 (1977) 53–55.
15 M. A. Klisenko and L. G. Alexandrova, in M. A. Klisenko (Editor), *Metody Opredeleniya Mikrokolichestv pesticidov v Produktakh Pitaniya, Kormakh i Vneshnej Sredye*, Kolos, Moscow, 1977.
16 E. G. Ivaniyk, M. D. Babina and V. G. Ovechkin (Editors), *Metodicheskiye Ukazaniya na Metody Opredeleniya Vrednykh Veschestv v Vozdukhe*, Vol. XV, MZO SSSR, Moscow, 1979.

Journal of Chromatography, 247 (1982) 263–272
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,076

GAS CHROMATOGRAPHIC ANALYSIS OF HALOCARBONS IN DRINKING WATER BY HEADSPACE EXTRACTION AND MIXED COLUMN SEPARATION

G. CASTELLO* and T. C. GERBINO
Istituto di Chimica Industriale, Università di Genova, Corso Europa 30, 16132 Genova (Italy)
and
S. KANITZ
Istituto di Igiene, Università di Genova, Via Pastore 1, 16132 Genova (Italy)
(Received May 4th, 1982)

SUMMARY

Series arrangement of polar and non-polar liquid stationary phases (30 cm of 10% SP-1000 on 80–100-mesh Chromosorb W DMCS, followed by 350 cm of 10% OV-1 on the same support) were used for the complete resolution of the trihalomethanes $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$ and $CHBr_3$, formed in drinking water facilities by the action of the chlorine used as a disinfectant. Other halocarbons ($CCl_2=CH_2$, CH_2Cl_2, $CHCl_2CH_3$, CCl_3CH_3, CCl_4, $CHCl=CCl_2$, $CCl_2=CCl_2$) not formed during chlorination but coming from polluted water sources, which interfere in the analysis of trihalomethanes due to peak overlap, can be separated with the same column arrangement, and are eluted in the order listed. Homogeneously mixed columns with similar polar–non-polar liquid phase ratios were also tested but showed lower resolving powers and required a more complex preparation method.

A headspace extraction technique and a ^{63}Ni electron-capture detector were used for quantitation. With proper temperature programming of the column, gas chromatographic analysis could be completed within 30 min, making this technique suitable for the rapid screening of large numbers of samples coming different water supplies in order to monitor the quality of community drinking water. Lack of interferences between the halocarbon peaks and those of commonly used liquid–liquid extraction solvents (*n*-pentane, *n*-hexane, isooctane) makes the proposed column also suitable for liquid exchange based trihalomethane extraction techniques.

INTRODUCTION

Chlorine has been used as a disinfectant for drinking water supplies for several decades, and until fairly recently its use has never been questioned. In 1974 it was reported[1,2] that chlorine reacts with organic precursors occurring in raw water to produce a potential carcinogen, chloroform ($CHCl_3$), together with other trihalomethanes (THMs): $CHBrCl_2$, $CHBr_2Cl$ and $CHBr_3$[3–6]. In addition, the increasing use of

0021-9673/82/0000–0000/$02.75

chlorinated hydrocarbons in industrial solvents increased the possibility of pollution of rivers, lakes and well waters with halogenated compounds that cannot be easily removed during standard purification treatments.

Many analytical methods have been suggested for the determination of THMs in drinking water, and EPA official methods are also available. Method 501.1[7] is a procedure for the analysis of THMs by the purge and trap technique while method 501.2[8] is based on liquid–liquid extraction. Headspace techniques[9–16] with manual or automatic sampling was found to be a useful method for the rapid screening of large numbers of water samples from different supplies, in order to monitor the quality of community drinking water utilities. The simplicity of the manual headspace extraction technique and its easy application by different laboratories was the reason for the choice of this method for the wide monitoring of the concentration of THMs in the water systems of various Italian towns, sponsored by the Italian Public Health Ministry and the Istituto Superiore di Sanità, Rome. All of the laboratories involved used the technique fully described in refs. 14 and 17 and the results will be published officially in the near future.

Owing to the possibility of halocarbons other than THMs from chlorination that could be present in raw waters, and which have identical retention times on a particular column, two liquid stationary phases were chosen for use in combination: OV-101 (non-polar methylsilicone) and SP-1000 (polar polyethylene glycol Carbowax 20M plus terephthalic acid). Table I shows the McReynolds constants[18] of these two phases and of similar columns previously used for the separation of chlorinated hydrocarbons[5,14,17,19–29], which lie near to the opposite extremes of the McReynolds polarity scale.

TABLE I

McREYNOLDS CONSTANTS OF NON-POLAR AND POLAR LIQUID STATIONARY PHASES USED FOR GC SEPARATION OF THMs

Stationary phase	*McReynolds constants*					*Ref.*
	ΔI benzene (x′)	*ΔI butanol (y′)*	*ΔI 2-pentanone (z′)*	*ΔI nitropropane (v′)*	*ΔI pyridine (s′)*	
Squalane	0	0	0	0	0	28
SE-30	15	53	44	64	41	25
OV-1	16	55	44	65	42	17,23,24
OV-101	17	57	45	67	43	21,26
SP-2100	17	57	45	67	43	14,28
Apiezon L	32	22	15	32	42	19
Di(2-ethylhexyl) sebacate	72	168	108	180	123	15
OV-11	102	142	145	219	178	28
OV-17	119	158	162	243	202	26
Tricresyl phosphate	176	321	250	374	299	22
OV-210	146	238	358	468	310	25
Carbowax 20M	322	536	368	572	510	25,27,22
Carbowax 1500	347	605	418	626	589	14
SP-1000	332	355	393	583	546	17,28,29
FFAP	340	580	397	602	627	17,25,27
TCEPE	526	782	677	920	837	20

Injection of the sample on both columns was necessary because the non-polar phase could not separate 1,2-dichloroethane from carbon tetrachloride or trichloroethylene from dichlorobromomethane, and the polar phase showed identical retentiontion times for carbon tetrachloride and 1,1,1-trichloroethane. As a consequence, the advantage, in terms of analysis time, due to the rapid injection technique of the headspace method was lost owing to the need to repeat the analysis on two columns. When a dual-column instrument with two electron-capture detectors (ECDs) is available, simultaneous analyses could be accomplished on the two columns mounted in parallel, but this solution is expensive, as two complete analytical lines are necessary (ECD, amplifier, recorder and data system), and some correction factors have to be taken into account because two samples are extracted from two different vials, and the standing currents of the ECDs may be different on the two sides of the instrument. When a parallel arrangement of the two columns is not available, a complete set of analyses has to be repeated on the polar column, installed in the oven to replace the non-polar column, and additional time is lost waiting for ECD stabilization.

Therefore, a method was developed which, by using a polar and a non-polar column mounted in series and connected to a ^{63}Ni ECD, permitted the separation, by temperature programming, of all of the halocarbons that could be present in the samples.

Pre-columns were previously used in the purge and trap method[28] but they were filled with a higher concentration of the same phase used in the main column, and did not change the order of elution of the compounds. A small amount (0.1 %) of Carbowax 1500 was also mixed with the 20 % of the main liquid phase SP-2100[14], and probably had the function of a tail reducer. A mixed phase typically tailored for pesticide analysis (6 % OV-210 + 4 % SE-30) has also been used[25].

EXPERIMENTAL

The headspace technique described in refs. 14 and 17 was used with some modifications. Screw-capped vials of volume 40 ml with PTFE-faced septa, washed with distilled water and methanol and dried overnight at 200°C, were used; the caps and septa were washed with light petroleum and dried overnight at 80°C; laboratory glassware was washed with distilled water and methanol and dried at 300°C.

The addition of sodium chloride to the standards and to the samples that had to be analysed immediately was effected by adding 4 ml of 40 % sodium chloride solution in doubly distilled water to each vial before the final drying at 200°C. This procedure removed all traces of organic substances and avoided contamination due to salt addition during the sample pick-up. This procedure was not followed during the preparation of samples that had to be analysed 24 and 72 h after sampling in order to study the formation of THMs due to long contact of precursors with free chlorine[30], because of the influence of sodium chloride and its impurities on the formation of bromine derivatives. Sodium thiosulphate solution (*ca.* 150 mg/l) was added to remove residual chlorine immediately after sample pick-up (for samples that had to be analysed at time zero) and before analysis (for samples analysed after 24 and 72 h). The vials were completely filled with the sample, tightly sealed and stored in the dark at 15°C.

Before analysis, 5 ml of water were removed from each vial in order to create the headspace volume, and the samples were thermostated at 30°C for 1 h in a water-bath. The injections (200 μl) were made by using 250-μl Hamilton gas-tight syringes.

Stainless-steel columns (3 m × $\frac{1}{8}$ in. O.D.) were used at 60°C for the isothermal analysis on polar and non-polar stationary phases (10% of SP-1000 and 10% of OV-1 on Chromosorb W DMCS, 80–100 mesh). Mixed columns were prepared by homogeneously mixing suitable amounts of the packings. Series arrangements of the two phases were accomplished by filling a single column with different lengths of the two coated supports or by using separate columns connected by means of standard Swagelock unions (1/8 in. to 1/8 in., stainless steel). The resulting small dead volume between the two columns could be reduced by drilling a hole with the same diameter as the tubing through the union, and facing the two columns closely end-to-end. The effect of this volume reduction on the plate height was negligible for total column lengths greater than 1.5 m.

A Varian 3700 gas chromatograph, equipped with a ^{63}Ni (8 mCi) ECD was used for experiments with mixed columns and linear temperature programming. Isothermal analysis with a parallel column arrangement was also carried out with a Varian 660 dual-column, dual-ECD (250 mCi tritium each) gas chromatograph. Quantitative analysis was accomplished by manual integration or by using Varian CDS-111C and Vista 401 data systems. The external standard technique, by injection of the headspace volumes from vials with known contents of each halocarbon, was used for calibration.

RESULTS AND DISCUSSION

The choice of the optimum ratio between polar and non-polar stationary phases in the column was made on the basis of the retention times obtained on columns filled with the two phases (10% each). Table II shows adjusted retention times, t'_R, on pure OV-1, pure SP-1000 and 1:1 mechanically blended packings. The widely accepted hypothesis that this type of mixed-bed packing yields retentions that are linear as a function of column composition[31] was confirmed to a reasonable

TABLE II

ADJUSTED RETENTION TIMES, t'_R (min), OF HALOGENATED COMPOUNDS ON PURE OV-1, PURE SP-1000 AND 1:1 MIXED PHASES

Temperature, 60°C; carrier gas flow-rate, 25 cm^3/min.

Compound	*OV-1*	*SP-1000*	*OV-1–SP-1000 (1:1)*
Methylene chloride	2.32	5.85	4.10
Chloroform	4.1	11	7.6
1,1,1-Trichloroethane	5.4	4.1	4.6
Carbon tetrachloride	6.4	4.1	5.0
Trichloroethylene	8.3	9.0	8.3
Dichlorobromomethane	8.3	28.4	18.8
Dibromochloromethane	16.6	81.1	47.2
Tetrachloroethylene	20.1	11.1	14.7
Bromoform	29	184	100

extent, because the t'_R values obtained on the mixed column are near to the straight lines connecting the t'_R values given by the pure packings in separate columns. Mechanically mixed columns having different polar–non-polar ratios permitted a more accurate dependence of the retention on column composition to be calculated, which is reported as retention relative to trichloroethylene (r) in Fig. 1. The relative retention values are independent of small changes in carrier gas flow-rate and temperature, and therefore permit an easier comparison of results obtained with different columns, showing that complete separation of the considered compounds is impossible with either pure stationary phase, because of overlapping of some peaks, whereas complete resolution may be obtained by using a suitable mixture of the two packings.

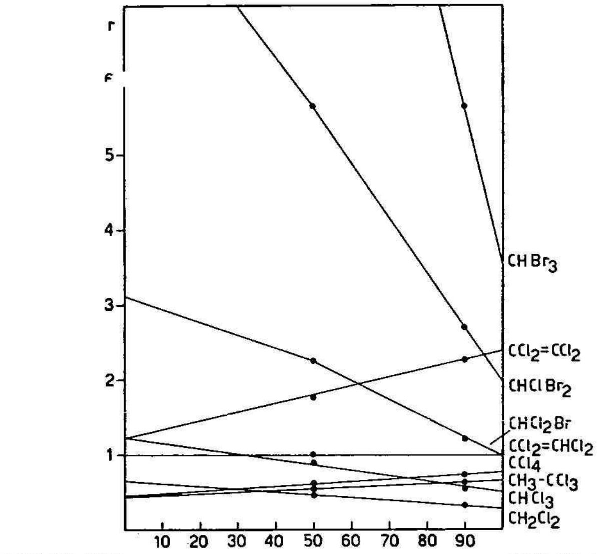


Fig. 1. Relative retention (r) with respect of trichloroethylene as a function of column composition. Data for pure SP-1000, pure OV-1, a 1:1 mixture and a 1:10 mixture were obtained experimentally. Lines are interpolated.

In order to obtain both complete resolution and short analysis times, mixed stationary phases with a large excess of non-polar OV-1 were preferred. In this situation, mechanical mixing would ensure a homogeneous distribution of the two phases along the column only after prolonged, vigorous shaking. As this could crush the packing particles with adverse effects on its mesh size distribution, surface deactivation and impregnation, the series arrangement of two separate columns may be used.

When series-connected columns are used instead of homogeneously mixed packings, the effect of the upstream column is increased, because it operates between the inlet pressure, P_i, of the system and the pressure at the connection point, P_c, which differs from the oulet pressure owing to the flow resistance of the downstream column. It was shown[32] that the contribution of the upstream column to the total retention time is equivalent to the values measured when the same column is operated alone between P_i and P_c. The results therefore differ, depending on the order of installation of the two columns, as shown in Table III, where the values of r obtained with direct and reversed connection of 30 cm of polar and 300 cm of non-polar column are given. Resolution values, calculated as $R = 2\,d/(w_1 + w_2)$, where d is the distance between the tops of two consecutive peaks and w_1 and w_2 are their base widths, are also reported and show that the best result is obtained when the polar section of the column is upstream.

TABLE III

RETENTIONS RELATIVE TO TRICHLOROETHYLENE (r) OF HALOGENATED COMPOUNDS ON PURE STATIONARY PHASES AND HOMOGENEOUSLY MIXED (1:1) AND SERIES (10:1) COLUMNS

Resolutions, R, are also reported (for series arrangement with non-polar and polar columns, respectively, upstream) between each peak and the following peak. Temperature, 60°C.

Compound	*OV-1*	*SP-1000*	*OV-1–SP-1000 (1:1)*	*OV-1–SP-1000 (10:1)*			
				OV-1 upstream		*SP-1000 upstream*	
	r	*r*	*r*	*r*	*R*	*r*	*R*
Methylene chloride	0.28	0.65	0.50	0.33	5.0	0.33	3.25
Chloroform	0.49	1.23	0.91	0.54	1.6	0.54	0.38
1,1,1-Trichloroethane	0.65	0.45	0.55	0.63	1.1	0.63	1.30
Carbon tetrachloride	0.77	0.45	0.60	0.74	2.15	0.74	2.27
Trichloroethylene	1.0	1.0	1.0	1.0	0.72	1.0	1.66
Dichlorobromomethane	1.0	3.15	2.26	1.15	5.49	1.23	5.43
Dibromochloromethane	2.0	9.01	5.68	2.27	0.076	2.72	7.4
Tetrachloroethylene	2.42	1.23	1.77	2.42	6.4	2.29	1.44
Bromoform	3.49	20.5	12.04	5.16	–	5.48	–

By taking into account the different effects of the same column working at the higher pressure of the upstream section, a small correction to the two lengths of the polar and non-polar stationary phases should be applied, by increasing the non-polar section. In order to avoid a time-consuming trial-and-error method, a window diagram[33] was drawn by plotting as a function of the column composition the α values for these of the $[n!/2(n - 2)!]$ pairs of solutes that show peak interference. α was calculated as

$$\alpha_{j/i} = \frac{t'_{R_j}}{t_{R_i}} \text{ or } \alpha_{j/i} = \frac{r_j}{r_i}$$

In Fig. 2, α is everywhere maintained greater than or equal to unity by inversion where necessary of the i/j assignment of each pair of solutes. Several windows (shaded regions) formed by intersection of lines of α indicate the phase composition range where the separation of a given pair can be achieved. On the basis of these results, a column containing 0.7% of SP-1000 and 9.3% of OV-1 should be the best choice in order to obtain complete separation. This polar–non-polar ratio was achieved by connecting 30 cm of 10% SP-1000 with 350 cm of 10% OV-1 or by filling a single column with a mechanical mixture of 7 parts of the polar and 93 parts of the non-polar phase. For both columns, the minimum of the HETP values of the Van

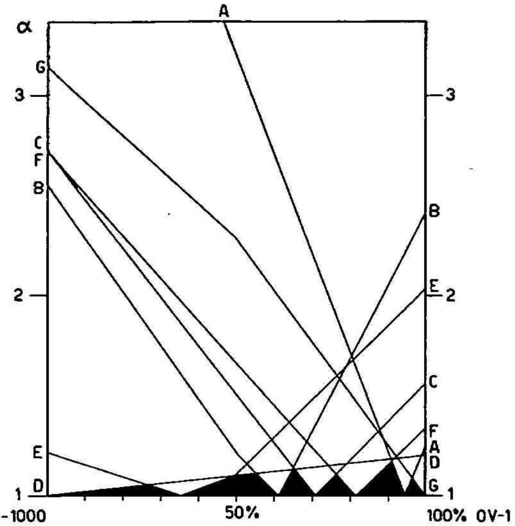


Fig. 2. α values of solvent pairs that show interference as a function of mixed column composition. A, Dibromochloromethane–tetrachloroethylene; B, tetrachloroethylene–dichlorobromomethane; C, chloroform–carbon tetrachloride; D, carbon tetrachloride–1,1,1-trichloroethane; E, chloroform–trichloroethylene; F, chloroform–1,1,1-trichloroethane; G, trichloroethylene–dichlorobromomethane.

Deemter equation[34,35] was found to lie in the nitrogen flow-rate range 16–20 cm^3/min, which agrees with those required for a satisfactory sensitivity of the ^{63}Ni ECD.

At 60°C, the resolution was practically the same on both series and mixed phases having the same polar–non-polar ratio (Table IV). By increasing the temperature in order to decrease the time of analysis, the best resolution was obtained for the series arrangement at 78°C, while the $CHCl_2{=}CHCl_2$ and $CHBr_2Cl$ peaks overlapped on the homogeneously mixed phase.

In order to compensate for the prevailing influence of the upstream polar section in the series system, small variations of the composition of the mixed phase were tested (Table IV). Mixed columns containing 9% of OV-1 and 1% of SP-1000 increased the resolution between $CHCl_2{=}CHCl_2$ and $CHBr_2Cl$, but yielded very close retention times for $CHCl_3$ and CCl_3CH_3, which could not be separated when one of the two compounds was in excess with respect to the other.

Therefore, the series arrangement gives better results than the theoretically equivalent homogeneously mixed columns, as it permits, at any temperature, the separation of THMs and other halocarbons (Fig. 3). Also 1,1-dichloroethane and 1,1-dichloroethylene can easily be resolved (r = 0.38 and 0.24, respectively).

As the aim of this work was mainly the reduction of the analysis time, further attempts in this direction were made by temperature programming. Fig. 3 shows the chromatogram obtained with the best combination of the initial and final isothermal temperature and programming rate, and Table IV shows the t'_R and r values. Temperature programming could only be used with ^{63}Ni ECD, because the baseline drift of this detector with temperature was negligible with respect to the ^3H ECD. The rise of the baseline during programming was small enough to permit easy quantitation

TABLE IV

ADJUSTED RETENTION TIMES (t'_R) AND RETENTION RELATIVE TO TRICHLOROETHYLENE (r) ON SERIES COLUMNS (30 cm OF 10% SP-1000 UPSTREAM AND 350 cm OF 10% OV-1 DOWNSTREAM) AND HOMOGENEOUSLY MIXED COLUMNS OF DIFFERENT COMPOSITIONS

Carrier gas flow-rate, 18 cm^3/min. Temperature programme: 9 min at 78°C, increased at 5°C/min to 110°C.

n*	*Compound*	*Series columns* (60°C)		*Mixed column (9.3%) OV-1–0.7% SP-1000* (60°C)		*Series columns* (78°C)		*Mixed column (9.3%) OV-1–0.7% SP-1000)* (78°C)		*Mixed column (9% OV-1–1% SP-1000)*				*Mixed column (9.15% OV-1–0.85% SP-1000)*				*Series column temperature programming)*	
										60°C		78°C		60°C		78°C			
		t'_R	r	t'_R	r	t'_R	r	t'_R	r	t'_R	r	t'_R	r	t'_R	r	t'_R	r	t'_R	r
–	1,1-Dichloroethylene	–	–	–	–	2.20	0.24	–	–	–	–	–	–	–	–	–	–	2.20	0.24
0	Methylene chloride	5.5	0.3	3.4	0.30	2.58	0.28	2.1	0.32	2.76	0.25	2.10	0.34	2.50	0.25	1.7	0.27	2.58	0.28
–	1,1-Dichloroethane	–	–	–	–	3.52	0.38	–	–	–	–	–	–	–	–	–	–	3.52	0.38
1	Chloroform	9.25	0.56	6.3	0.55	5.36	0.58	3.6	0.56	6.30	0.58	3.55	0.58	5.3	0.53	3.4	0.55	5.36	0.59
2	1,1,1-Trichloroethane	10.2	0.61	7.2	0.63	5.98	0.64	4.3	0.67	6.70	0.61	3.65	0.60	6.3	0.63	4.1	0.66	5.98	0.66
3	Carbon tetrachloride	11.8	0.72	8.4	0.73	6.92	0.75	5.0	0.78	7.88	0.72	4.60	0.75	7.4	0.75	4.7	0.76	6.92	0.76
4	Trichloroethylene	16.5	1.00	11.4	1.00	9.23	1.00	6.4	1.00	10.87	1.00	6.11	1.00	9.9	1.00	6.2	1.00	9.10	1.00
5	Dichlorobromomethane	20.4	1.23	13.5	1.36	10.94	1.18	7.4	1.15	13.85	1.27	7.30	1.20	11.2	1.31	6.9	1.1	10.48	1.15
6	Tetrachloroethylene	38.6	2.30	26.9	2.42	20.34	2.20	14.34	2.20	25.72	2.36	13.40	2.19	23.5	2.37	13.8	2.22	15.28	1.68
7	Dibromochloromethane	45.9	2.80	28.9	2.53	22.37	2.42			30.04	2.76	14.82	2.42					16.30	1.79
8	Bromoform	100	6.05	67.2	5.90	46.24	5.01	32.0	5.0	–	–	30.1	4.92	–	–	–	–	24.41	2.68

* Refers to peaks in Fig. 3.

with both manual and electronic (Varian CDS-111 C) integration. As the baseline deviation is quite reproducible owing to the thermal stability of the liquid phase used, it can easily be stored and substracted by suitable computer programs or by using GC-dedicated data systems (Varian Vista 401 equipped with at least one floppy disk drive unit); ten samplings per minute of the baseline level permitted nearly complete suppression of the baseline deviations in the plotted chromatogram.

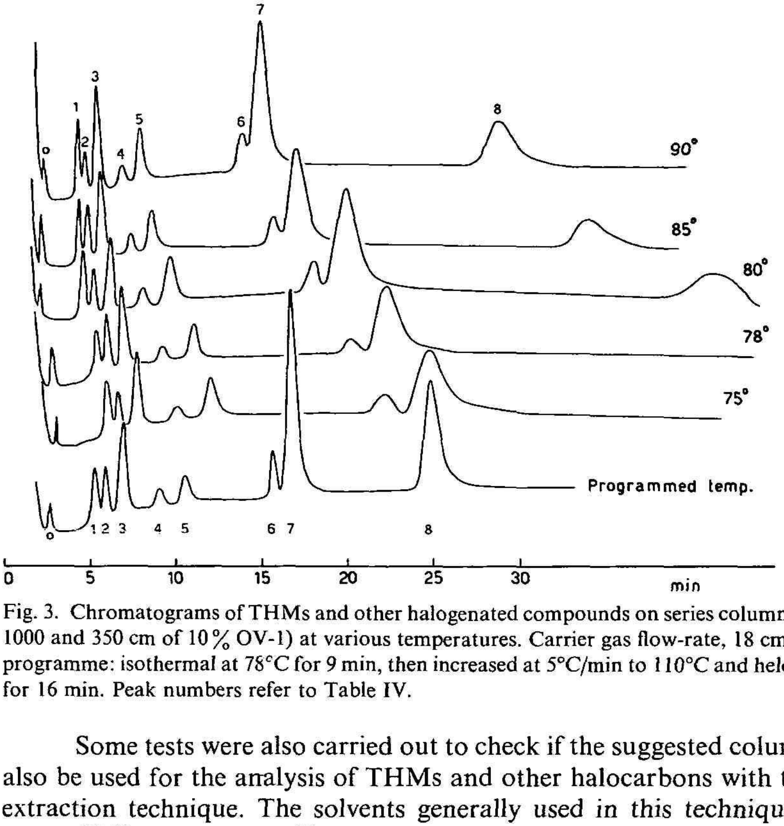


Fig. 3. Chromatograms of THMs and other halogenated compounds on series columns (30 cm of 10% SP-1000 and 350 cm of 10% OV-1) at various temperatures. Carrier gas flow-rate, 18 cm^3/min. Temperature programme: isothermal at 78°C for 9 min, then increased at 5°C/min to 110°C and held at this temperature for 16 min. Peak numbers refer to Table IV.

Some tests were also carried out to check if the suggested column system could also be used for the analysis of THMs and other halocarbons with the liquid–liquid extraction technique. The solvents generally used in this technique, *n*-pentane, *n*-hexane[25,29] and isooctane[25], were injected together with a complete standard mixture of the halocarbons listed in Table II. *n*-Pentane was eluted before all of the chlorinated compounds, *n*-hexane between methylene chloride and chloroform and isooctane between carbon tetrachloride and trichloroethylene.

Owing to the specific response of the ECD, peaks corresponding to the injection of 4 μl of hydrocarbons had the same shape and area as those due to the amounts of halogenated compounds found in drinking waters, and little interference was therefore found. Of course, liquid–liquid extraction requires "ECD pure" solvents, and some problems can be caused by the extraction of non-volatile ECD-sensitive compounds present in the sample that are not detected with the headspace technique.

CONCLUSIONS

The suggested arrangement of polar and non-polar stationary phases, in connection with a proper temperature programming, permitted the time needed for complete analysis of each headspace sample to be reduced to less than 30 min. This increases the usefulness of the headspace technique for the rapid and wide screening of water supplies in order to monitor the amount of THMs and other volatile halocarbons. The columns and conditions described also permit the analysis of samples with the liquid–liquid extraction technique.

The series configuration is more efficient than a homogeneously mixed column with a similar polar–non-polar ratio, as the upstream position of the polar phase assures that its effect predominates during the initial isothermal stage of temperature programming, thus enhancing the resolution of low-boiling compounds. Small adjustments of the retention times of some peaks in complex mixtures can be obtained by slightly changing the length of the polar section, whereas a cumbersome trial-and-error method must be used for satisfactory modification of the homogeneously mixed columns.

REFERENCES

1 J. J. Rook, *Water Treat. Exam.*, 22 (1974) 234.
2 T. A. Bellar, J. J. Litchtenberg and R. C. Kroner, *J. Amer. Water Works Ass.*, 66 (1974) 703.
3 J. J. Rook, *J. Amer. Water Works Ass.*, 68 (1976) 168.
4 J. J. Rook, *Environ. Sci. Technol.*, 11 (1977) 478.
5 J. J. McCreary and V. L. Snoeyink, *Environ. Sci. Technol.*, 15 (1981) 193.
6 V. L. Snoeyink, R. R. Clark, J. J. McCreary and W. F. McHie, *Environ. Sci. Technol.*, 15 (1981) 189.
7 *The Analysis of Trihalometanes in Finished Waters by the Purge and Trap Method*, Method 501.1, USEPA, EMSL, Cincinnati, OH, 1979.
8 *The Analysis of Trihalomethanes in Drinking Water by Liquid/Liquid Extraction*, Method 501.2, USEPA, EMSL, Cincinnati, OH, 1979.
9 H. Binder, *J. Chromatogr.*, 25 (1966) 189.
10 F. Drawert, W. Heimann, R. Emberger and R. Tressl, *Chromatographia*, 2 (1969) 77.
11 B. Kolb, *J. Chromatogr.*, 122 (1976) 553.
12 H. Hachenberg and A. P. Schmidt, *Gas Chromatographic Headspace Analysis*, Heyden, London, New York, Rheine, 1977.
13 R. Olson, D. T. Williams and P. D. Bothwell, *Environ. Sci. Technol.*, 13 (1979) 936.
14 E. A. Dietz and K. F. Singley, *Anal. Chem.*, 51 (1979) 1609.
15 L. Lukačovič, M. Mikuláš, A. Vanko and G. Kiss, *J. Chromatogr.*, 207 (1981) 373.
16 A. Cavallaro and P. Grassi, *Boll. Lab. Chim. Prov.*, 27 (1976) 337.
17 G. Ziglio and G. Beltramelli, *Quad. Ist. Igiene, Univ. Milano*, No. 2, July 1980.
18 W. O. McReynolds, *J. Chromatogr. Sci.*, 8 (1970) 685.
19 G. Novari and G. Castello, *Rass. Chim.*, 4 (1970) 114.
20 G. Castello, G. Novari and P. Pedemonte, *Rass. Chim.*, 6 (1972) 367.
21 B. M. Austern, R. A. Dobbs and J. M. Cohen, *Environ. Sci. Technol.*, 9 (1975) 588.
22 G. Castello and G. D. D'Amato, *J. Chromatogr.*, 76 (1973) 293.
23 K. L. E. Kaiser and B. G. Oliver, *Anal. Chem.*, 48 (1976) 2207.
24 J. P. Mieure, *J. Amer. Water Works Ass.*, 69 (1977) 60.
25 J. Richard and G. A. Junk, *J. Amer. Water Works Ass.*, 69 (1977) 62.
26 D. J. Norwood, J. D. Johnson and R. F. Christman, *J. Amer. Chem. Soc.*, 14 (1979) 187.
27 H. Norin and L. Renberg, *Water Res.*, 14 (1980) 1397.
28 H. J. Brass, *Int. Lab.*, Sept. (1980) 17.
29 A. Kirshen, *Varian Instrum. Appl.*, 5 (1981) 2.
30 A. A. Stevens, C. S. Slocum, D. R. Seeger and G. R. Robeck, *Water Chlorination: Environmental Impact and Health Effects*, Vol. 1, Ann Arbor Sci. Publ., Ann Arbor, MI, 1978, p. 77.
31 C. F. Chien, M. M. Kopecni and R. J. Laub, *Anal. Chem.*, 52 (1980) 1042.
32 G. Castello and G. D'Amato, *Ann. Chim. (Rome)*, 69 (1979) 541.
33 R. J. Laub and J. H. Purnell, *J. Chromatogr.*, 112 (1975) 71.
34 J. J. Van Deemter, F. J. Zwiderweg and A. Klinkenberg, *Chem. Eng. Sci.*, 5 (1956) 271.
35 A. I. M. Keulemans, *Gas Chromatography*, Reinhold, New York, 1958.

Journal of Chromatography, 247 (1982) 273–280
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,071

GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF BENZETHONIUM CHLORIDE

SHŌZI KAWASE and SUSUMU KANNO
Identification Section, Gifu Prefectural Police Headquarters, 1, Yabuta-1-chome, Gifu 500 (Japan)
and
SHIGEO UKAI*
Gifu College of Pharmacy, 6-1, Higashi-5-chome, Mitahora, Gifu 502 (Japan)
(Received May 26th, 1982)

SUMMARY

Benzethonium chloride (I), used as a detergent, disinfectant and pharmaceutical preparation, was determined by gas–liquid chromatography of the reduction product obtained by treatment with sodium borohydride and nickel(II) chloride. The reduction product of I was identified as 2-{2-[*p*-(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy}ethyldimethylamine by mass spectrometry, nuclear magnetic resonance spectroscopy and elemental analysis. In the reduction, the benzyl group of I was eliminated as toluene. The procedure is suitable for the assay of 10–100 μg/ml of aqueous preparations of I, and useful for the routine analysis of I, as the reduction takes place quantitatively, the reagents are relatively safe to handle and there is no need for complicated apparatus such as in catalytic hydrogenation.

INTRODUCTION

During studies on the determination of quaternary ammonium compounds, we have studied the determination of benzethonium chloride, used as a detergent, disinfectant and pharmaceutical preparation, by gas–liquid chromatography (GLC). The analysis of benzethonium chloride has previously been performed by spectrophotometry[1–11], titrimetry[12–18], gravimetry[11,19–21] and thin-layer chromatography[22]. The disadvantage of these methods is the lack of specificity for the determination of benzethonium chloride. On the other hand, GLC of the benzethonium salt, which is non-volatile, has previously been carried out by a thermal decomposition method[23,24], and has not been used in quantitative analysis because of the appearance of multiple peaks and the poor response.

In previous papers, we reported that cationic surfactants based on N-alkylpyridinium salts[25] and N-alkyldimethylbenzylammonium salts[26] could be determined by GLC of their reduction products obtained by reduction with a mixture of sodium borohydride and a transition metal salt, *e.g.*, nickel(II) chloride. This paper deals with the GLC determination of benzethonium chloride by use of the reduction system of sodium borohydride ($NaBH_4$) and nickel(II) chloride ($NiCl_2$).

0021-9673/82/0000–0000/$02.75

EXPERIMENTAL

Apparatus

GLC was carried out with a glass column (1 m × 0.3 cm I.D.) on a Hitachi Model 073 gas chromatograph equipped with a hydrogen flame-ionization detector (HFID). Mass spectrometry was performed on a JEOL JMS-D300 mass spectrometer. Nuclear magnetic resonance spectroscopy (NMR) was carried out with a Hitachi Model R-20 (60 MHz) instrument.

Materials

All chemicals were of analytical-reagent grade. Freshly distilled diethyl ether was used in the extraction of the reduction products.

Benzethonium chloride (I) was commercially available.

Synthesis of 2-{2-[p-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy}ethyldimethylamine (II) from I by the $NaBH_4$–$NiCl_2$ reduction system

I (4.48 g, 0.01 mol) and $NiCl_2 \cdot 6H_2O$(2.38 g, 0.01 mol) were dissolved in methanol (300 ml). To the solution was added $NaBH_4$ (18.9 g, 0.5 mol) in small portions at 25°C with stirring, which was continued for 1 h at room temperature. A black precipitate was formed and hydrogen was evolved. The precipitate was filtered off and washed with methanol, and the filtrate and the washings were combined and concentrated to one third of the initial volume under reduced pressure. To the residue was added water (50 ml) and the mixture was extracted with *n*-hexane (three 50-ml volumes). The *n*-hexane layer was dried over sodium sulphate and evaporated to dryness. The resulting residue was distilled under reduced pressure to give a colourless oil, b.p. 168–181°C/4 mmHg, in 82.7% yield. Elemental analysis: Calculated for $C_{27}H_{35}O_2N$, C 74.72, H 10.97, N 4.36%; found, C 74.42, H 11.11, N 4.39%.

Detection of toluene in the reduction of I with the $NaBH_4$–$NiCl_2$ system

To a vial (5 ml) was added an aqueous solution (1 ml) of I (100 μg) and 0.02 *M* $NiCl_2$ (0.5 ml). The vial was sealed tightly with a double rubber stopper. The air phase was removed with a syringe, and then to the vial was added 2.6 *M* $NaBH_4$ (0.5 ml) with the syringe through the double rubber stopper. After 5 min, the gas (2 ml) generated together with hydrogen in the vial was withdrawn with a syringe, and injected into the gas chromatograph. GLC was carried out under the following conditions: (A) a glass column (2 m × 0.3 cm I.D.) packed with 25% silicone DC550 on C-22 (40–60 mesh) at 120°C with a nitrogen flow-rate of 30 ml/min, an injection port temperature of 170°C and attenuation $10^2 \times 8$); (B) the same sized column packed with 25% PEG 6000 on Chromosorb W AW DMCS (60–80 mesh) at 90°C with a nitrogen flow-rate of 30 ml/min, an injection port temperature of 150°C and attenuation $10^2 \times 8$. The volatile compound was identified as toluene by comparing its retention time in GLC with that of an authentic sample. The retention time was 4 min (A) and 4.6 min (B).

Reduction of I with the $NaBH_4$–$NiCl_2$ system on the gas–liquid chromatographic scale

To an aqueous solution (1 ml) of I (10–100 μg) was added 0.02 *M* $NiCl_2$ (0.5 ml) and 2.6 *M* $NaBH_4$ (0.6 ml) with stirring. The mixture turned black and hydrogen was evolved. The mixture was allowed to stand for 1 h at room temperature, and was then extracted with diethyl ether (four 2-ml volumes). The combined organic layer was dried over sodium sulphate, acidified with a few drops of acetic acid and evaporated to dryness under reduced pressure. The residue was dissolved in ethyl acetate (200 μl) and 1 μl of the solution was injected into the gas chromatograph.

GLC was performed on a glass column (1 m × 0.3 cm I.D.) packed with 5% potassium hydroxide plus 5% PEG 20M on Chromosorb W AW DMCS (60–80 mesh) at 200°C with a nitrogen flow-rate of 50 ml/min, an injection port temperature of 250°C and attenuation 1 × 8. The internal standard was 0.01% anthracene.

Influence of the amount of $NaBH_4$ on the reduction of I with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of I (1 mg; 2.2 μmol) was treated with 0.33 ml (6.6 μmol) of 0.02 *M* $NiCl_2$ and different amounts of 2.6 *M* $NaBH_4$ (aqueous solutions of 110, 220, 440, 660, 880 and 1100 μmol) at room temperature for 1 h. The resulting reduction product was subjected to GLC as described above.

Influence of the amount of $NiCl_2$ on the reduction of I with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of I (1 mg; 2.2 μmol) was treated with different amounts of 0.02 *M* $NiCl_2$ (aqueous solutions of 1.1, 2.2, 4.4, 6.6, 8.8 and 11.0 μmol) and 2.6 *M* $NaBH_4$ (0.42 ml) at room temperature for 1 h. The resulting reduction product was examined by GLC as described above.

Rate of reduction of I with the $NaBH_4$–$NiCl_2$ system

An aqueous solution (1 ml) of I (1 mg; 2.2 μmol) was treated with 0.02 *M* $NiCl_2$ (6.6 μmol; 0.33 ml) and 2.6 *M* $NaBH_4$ (1.1 mmol; 0.42 ml) at room temperature for various times (15 min, 30 min, 1 h, 2 h and 3 h). The resulting reduction product were examined under GLC conditions as described above.

RESULTS AND DISCUSSION

Previously we have reported that the reduction of N-alkylpyridinium salts (detergent and disinfectant) and N-alkylbipyridylium salts (herbicide) with $NaBH_4$ alone gave the corresponding cyclic tertiary amines[25,27] containing a few double bonds, but the reduction of these quaternary ammonium salts with $NaBH_4$ and $NiCl_2$ afforded the corresponding expected reduction products, *viz.*, N-alkylpiperidines[25] and N-alkylbipiperidines[28,29]. Further, we have observed[26] that N-alkyldimethylbenzylammonium chloride (benzalkonium chloride: detergent and disinfectant) could not react with $NaBH_4$ alone, but treatment of the ammonium chloride with $NaBH_4$ and $NiCl_2$ gave N-alkyldimethylamine with reductive elimination of its benzyl group. Using a similar procedure to that reported previously, the $NaBH_4$–$NiCl_2$ reduction system was applied to the GLC determination of benzethonium chloride (I). The reduction product (II) thus prepared by treatment of I with $NaBH_4$–$NiCl_2$ was chromatographed in a glass column packed with 5% potassium hydroxide plus 5% PEG 20M on Chromosorb W AW DMCS at 200°C. As shown in Fig. 1, II is readily detected as a symmetrical peak.

Satisfactory separations were achieved on an alkaline column such as 5%

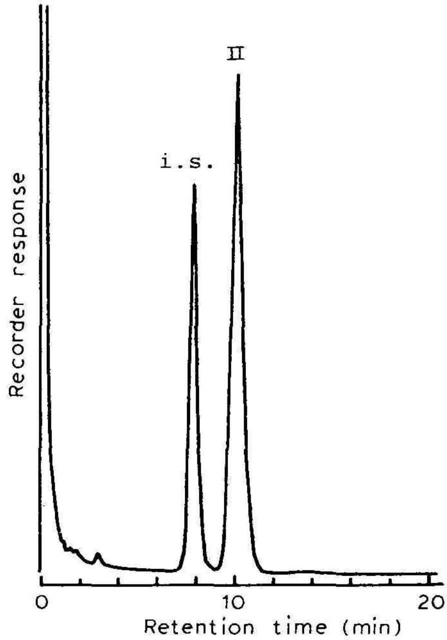


Fig. 1. Gas chromatogram of the hydrogenated product (II) derived from benzethonium chloride (I). Internal standard (i.s.): anthracene.

potassium hydroxide plus 5% PEG 20M, but neutral columns such as PEGS, PEG 20M, SE-30 and OV-17 exhibited slight tailing under isothermal conditions.

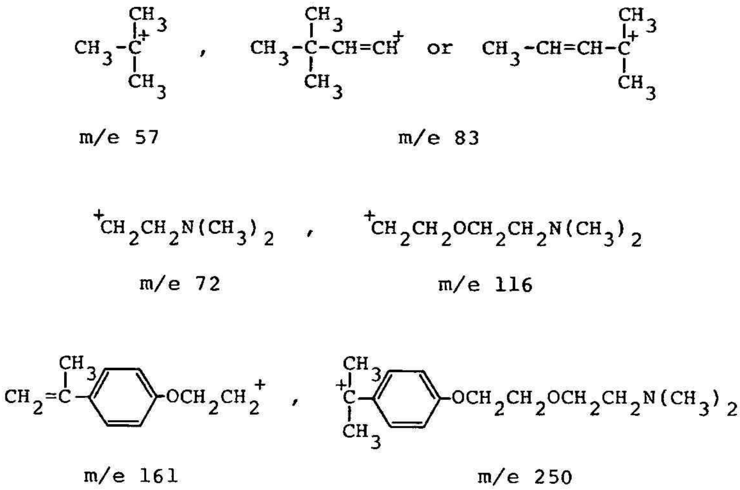
The structure of the reduction product (II) was clarified by an independent synthesis in the following manner. When a large excess of $NaBH_4$ was added to a methanolic solution of I and $NiCl_2$, a black precipitate of nickel boride was immediately formed, with evolution of hydrogen. The reduction of I proceeded smoothly with the continuous evolution of hydrogen, and was complete in about 1 h to afford the hydrogenated product (II), which was purified by distillation under reduced pressure to give a colourless oil.

The mass spectrum of II exhibited a base fragment peak at *m/e* 58 due to the N-dimethylaminomethylene and/or methylene oxyethylene ions, and also showed a parent ion peak at *m/e* 321 and several fragment ion peaks as indicated in Scheme 1.

The proton nuclear magnetic resonance of II in $CDCl_3$ exhibited $-C(CH_3)_3$ protons at δ 0.70 ppm (9H, singlet), $>C(CH_3)_2$ protons at δ 1.33 ppm (6H, singlet), $-\overset{|}{C}CH_2\overset{|}{C}-$ protons at δ 1.70 ppm (2H, singlet), $-N(CH_3)_2$ protons at δ 2.26 ppm (6H, singlet), $-OCH_2CH_2O-$ protons at δ 3.96 ppm (4H, multiplets), $>NCH_2CH_2O-$ protons at δ 2.25 ppm (2H, triplets) and δ 3.65 ppm (2H, triplets), aromatic protons at δ 6.82 ppm (2H, doublets) and δ 7.26 ppm (2H, doublets), but not exhibited protons due to the benzyl group.

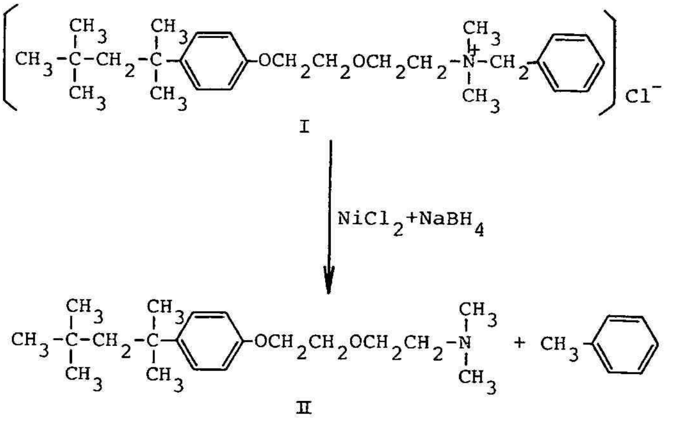
Based on these results and elemental analysis, the reduction product was identified as II.

The volatile compound generated together with hydrogen, except for II, in this reaction of I was identified as toluene by GLC using 25% silicone DC550 and 25% PEG 6000 columns on the basis of the experiment carried out in a vial. This demon-



Scheme 1.

strates that the reduction of I with $NaBH_4$ and $NiCl_2$ gave II with the release of a benzyl group from the molecule, as shown in Scheme 2. Consequently, it is clear that no methyl group is eliminated in this reduction. On the other hand, such a reduction did not proceed on treatment of I with $NaBH_4$ alone. These findings are similar to those of our earlier results[26] relating to N-alkyldimethylbenzylammonium chloride.



Scheme 2.

The conditions for the hydrogenation of I with $NaBH_4$ and $NiCl_2$ on the analytical (GLC) scale were examined in its aqueous solution. As shown in Fig. 2, the reduction of an appropriate amount of I (2.2 μmol in 1 ml of water), which corresponds to a high response in the determination, is dependent on the amount of $NiCl_2$ in the presence of a large excess of $NaBH_4$ (1.1 mmol in 0.43 ml of water). The hydrogenation of I to II proceeded quantitatively when more than 2.2 μmol of $NiCl_2$ (1.0 mol per mole of I) were used in the reduction system. In the presence of a definite

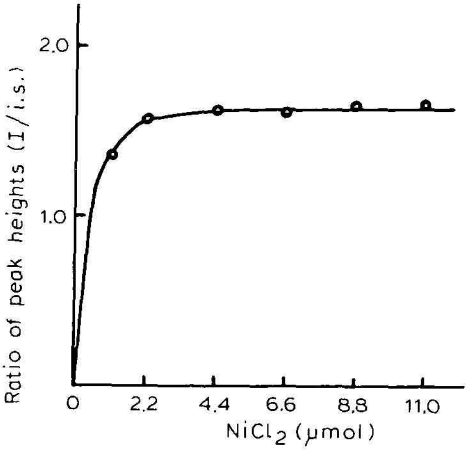
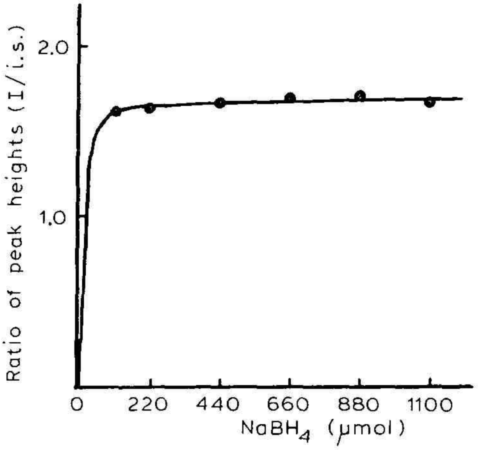


Fig. 2. Influence of amount of $NiCl_2$ on reduction of I to II with the $NaBH_4$–$NiCl_2$ system. Internal standard (i.s.): anthracene.

amount of $NiCl_2$ (6.6 µmol), the reduction was complete with amounts of $NaBH_4$ in the range 111–1115 µmol, as shown in Fig. 3. To avoid incomplete reduction, a large excess of reducing agent, such as the combination of 0.5 ml (10 µmol) of 0.02 *M* $NiCl_2$ and 0.6 ml (1.56 mmol) of 2.6 *M* $NaBH_4$, is advisable for the GLC analysis of I. Fig. 4 demonstrates the hydrogenation under these conditions is complete within 1 h at room temperature.



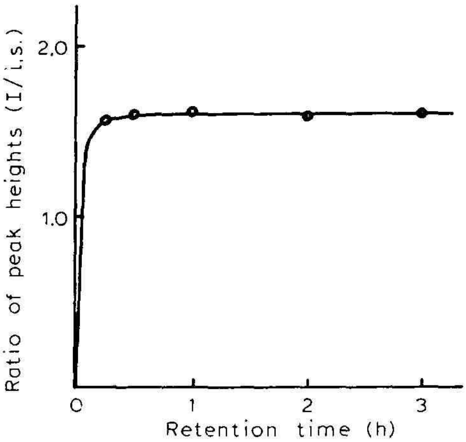


Fig. 3. Influence of amount of $NaBH_4$ on reduction of I to II with the $NaBH_4$-$NiCl_2$ system. Internal standard (i.s.): anthracene.

Fig. 4. Reduction of I with the $NaBH_4$-$NiCl_2$ system. Internal standard (i.s.): anthracene.

The determination of I under isothermal conditions was performed by the peak-height ratio method. The calibration graph for I (Fig. 5) showed good linearity using anthracene as the internal standard.

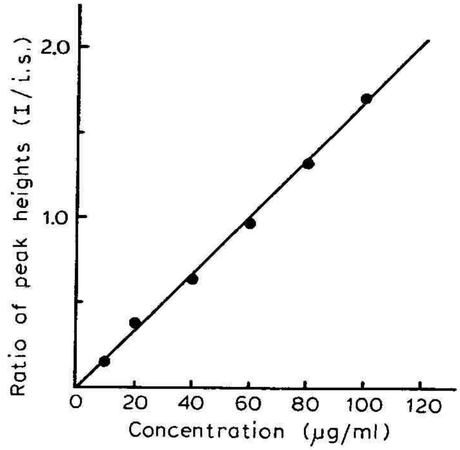


Fig. 5. Calibration graph for I. Internal standard (i.s.): anthracene.

The determination of I in several commercial preparations was carried out by this GLC method with satisfactory results, as shown in Table I.

TABLE I

DETERMINATION OF BENZETHONIUM CHLORIDE IN COMMERCIAL PREPARATIONS

*Sample No.**	*Aqueous solution of benzethonium chloride (%, w/v)*	*Recovery (%)*
1	10	91.3
2	10	97.4
3	4	91.4

* Samples: 1 = disinfectant; 2 = disinfectant containing 10% alkyl allyl polyether alcohol; 3 = gargle containing 0.47 ml/ml of ethanol and a flavouring.

CONCLUSION

The reduction of benzethonium chloride (I) with $NaBH_4$ and $NiCl_2$ proceeds complete to afford 2-{2-[*p*-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy}ethyldimethylamine (II) with the elimination of toluene, and I is readily determined by GLC of the reduction product (II). The procedure is suitable for the routine assay (10–100 μg/ml of aqueous preparation) of I, as the reduction takes place cleanly in aqueous medium at room temperature with easily handled reagents, without the need for complicated apparatus such as in catalytic hydrogenation. Further, the procedure would be applicable to the determination of other quaternary ammonium compounds in disinfectants and drugs.

REFERENCES

1 D. N. Parker, *Amer. Perfum. Cosmet.*, 85 (1970) 29; *C.A.*, 74 (1971) 15684c.
2 M. Pernarowski and L. G. Chatten, *J. Amer. Pharm. Ass.*, 47 (1958) 211.
3 R. L. Caswell, *J. Ass. Offic. Agr. Chem.*, 38 (1955) 304.
4 S. Tsurubou, N. Ohno and T. Sakai, *Nippon Kagaku Zasshi*, 53 (1980) 828.
5 J. B. Lowry, *J. Pharm. Sci.*, 68 (1979) 110.
6 L. G. Chatten and O. Okamura, *J. Pharm. Sci.*, 62 (1979) 1328.
7 M. Tsubouchi, *Bull. Chem. Soc. Jap.*, 44 (1971) 1560.
8 T. Chin and J. L. Lach, *J. Pharm. Sci.*, 54 (1965) 1550.
9 J. Birner, *Aust. J. Sci.*, 25 (1963) 416; *C.A.*, 59 (1963) 3718d.
10 H. Negoro and H. Sakurai, *Ann. Takamine Lab.*, 6 (1954) 89; *C.A.*, 49 (1955) 16473f.
11 K. Yoshimura and M. Morita, *Eisei Shikenjo Hōkoku*, No. 74 (1956) 77.
12 S. Pinzauti and E. La Porta, *Analyst (London)*, 102 (1977) 938.
13 M. Bares and J. Zajic, *Tenside Deterg.*, 11 (1974) 251; *C.A.*, 82 (1975) 45597j.
14 J. T. Cross, *Analyst (London)*, 90 (1965) 315.
15 F. Pellerin, J. A. Grautier and D. Demay, *Ann. Pharm. Fr.*, 22 (1964) 495; *C.A.*, 62 (1965) 12979f.
16 T. Uno, K. Miyajima and H. Tsukatani, *Yakugaku Zasshi*, 80 (1960) 153.
17 L. Worrell and W. Ebert, *Drug Stand.*, 24 (1956) 153.
18 J. A. Gautier, T. Rerault and F. Rellerin, *Ann. Pharm. Fr.*, 13 (1955) 725; *C.A.*, 50 (1956) 11884g.
19 D. M. Patel and R. A. Anderson, *Drug Stand.*, 26 (1958) 189; *C.A.*, 53 (1959) 14414b.
20 J. B. Wilson, *J. Ass. Offic. Agr. Chem.*, 35 (1952) 455; *C.A.*, 46 (1952) 11050b.
21 A. H. Tillson, W. V. Eisenberg and J. B. Wilson, *J. Ass. Offic. Agr. Chem.*, 35 (1952) 459.
22 S. M. Henry, G. Jacobs and H. Achmeteli, *Appl. Microbiol.*, 15 (1967) 1489; *C.A.*, 68 (1967) 19457e.
23 W. Bianchi, L. Boniforti and A. Di Domenico, *Mass Spectrom. Biochem. Med., Symp.*, 1973 (1973) 183; *C.A.*, 82 (1975) 35080w.
24 B. Tucci, C. Cardini, M. Cavazzutti and V. Quercia, *Boll. Chim. Farm.*, 107 (1968) 629.
25 S. Kawase, K. Hirose and S. Ukai, *J. Chromatogr.*, 213 (1981) 265.
26 S. Kawase and S. Ukai, *Eisei Kagaku*, 27 (1981) 296.
27 S. Ukai, K. Hirose and S. Kawase, *Eisei Kagaku*, 19 (1973) 281.
28 S. Ukai, K. Hirose and S. Kawase, *Eisei Kagaku*, 23 (1977) 32.
29 S. Ukai, K. Hirose and S. Kawase, *Eisei Kagaku*, 23 (1977) 83.

Journal of Chromatography, 247 (1982) 281–288
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,070

GEL CHROMATOGRAPHY OF OLIGOSACCHARIDES UP TO DP 60

MICHAEL JOHN*,*, JÜRGEN SCHMIDT*, CHRISTIAN WANDREY and HERMANN SAHM
Institut für Biotechnologie der Kernforschungsanlage Jülich, Postfach 1913, D-5170 Jülich 1 (G.F.R.)
(Received May 26th, 1982)

SUMMARY

Analytical and preparative gel chromatography have been used to separate oligosaccharides up to a degree of polymerization of 60 (fractionation range 180–10,000 daltons) using 40- and 47-μm polyacrylamide gel particles, respectively. Packing materials were obtained from commercial gels (Bio-Gel P-4 and P-6, −400 mesh) by an elutriation procedure. Columns were run at 60°C with water as eluent. The separation method has been applied to mixtures of malto-oligosaccharides, branched polymaltotrioses and xylo-oligosaccharides.

INTRODUCTION

Chromatography on polyacrylamide gel is one of the major analytical techniques for the separation and analysis of oligosaccharides. One of us previously described a method of fractionating malto-oligosaccharides up to a degree of polymerization (DP) of 15 on a Bio-Gel P-2 column at elevated temperature[1,2]. Several reports on the fractionation of oligosaccharides[3–7], oligosaccharide alditols[8] and uronic acid oligomers[9,10] using this procedure have since been published. Chromatography on Bio-Gel P-4 has been used for structural studies of glycoprotein-derived oligosaccharides[11,12]. Using polyacrylamide gels with different pore size, separations of oligosaccharides up to DP 25 were achieved.

Studies on the carbohydrate moiety of glycoproteins or glycolipids and the enzymic degradation of polysaccharides have made it necessary to introduce a gel chromatographic method that yields effective analytical and preparative separations of oligosaccharides containing more than 25 residues. We report here improved oligosaccharide separations up to DP 40 and 60 using carefully sized gel fractions of Bio-Gel P-4 and P-6, respectively.

EXPERIMENTAL

Chromatographic conditions

Bio-Gel P-4 (−400 mesh) and Bio-Gel P-6 (−400 mesh) were obtained from

---

* Present address: Max-Planck-Institut für Züchtungsforschung, Abt. Schell, D-5000 Köln 30 (Vogelsang), G.F.R.

Bio-Rad Labs. Since small and uniform particle sizes lead to more efficient separations as predicted by theory[13], the gels were fractionated in warm deionized water (*ca.* 50°C) by repeated settling and decanting the fines until a sharp zone of settling gel particles was attained. The elutriation procedure yielded mean particle sizes of the Bio-Gel P-4 and P-6 fractions of 40 ± 4 and 47 ± 4 μm, respectively. Prior to packing the column, a 50% gel slurry was degassed under vacuum. The column packing procedure was the same as described earlier[2]. In order to obtain a close gel bed, the rigid Bio-Gel P-4 was packed at a flow-rate of 20 $\text{ml} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$. With the softer P-6 gel only a slightly higher flow-rate than is to be used during operation should be applied for column packing.

All experiments were carried out with one home-made and two commercial columns. The column system for the Bio-Gel P-4 procedure consisted of two water-jacketed Cheminert columns (LC-1-43; 109 × 2.54 cm I.D.) connected in series with a zero dead volume union, so that the gel bed was 201 × 2.54 cm I.D. In order to avoid additional peak broadening, low dead volume connections (Cheminert fittings and adapters; PTFE tubing, 0.3 × 1.6 mm) were used. The column used for the Bio-Gel P-6 chromatography was similar to that described earlier[2], except that the column dimensions were 210 × 1.8 cm I.D. All columns were coated using a solution of 5% dichlorodimethylsilane in *n*-hexane[14].

The columns were operated at 60°C with degassed water (80°C) as eluent. In most experiments the flow-rate of the P-4 columns was 55 $\text{ml} \cdot \text{h}^{-1}$ ($\approx$ 1.4 bar) and of the P-6 column 25 $\text{ml} \cdot \text{h}^{-1}$ ($\approx$ 1.3 bar). Milton Roy minipumps were used for column elution. The two Bio-Gel P-4 columns were eluted in an upwards–downwards direction. For long-term use of the Bio-Gel P-6 column, upward flow elution was found to be advantageous. In order to obtain extreme purity of the water used as eluent, a column (20 × 1.5 cm I.D.; Econo-Column, Bio-Rad Labs.) filled with AG 501-X8D mixed bed ion-exchange resin and activated charcoal (Bio-Rad Labs.) was fitted between the pump and the eluent reservoir. The eluent was further filtered through a 0.8-μm Milli-Fil filter unit (Type PF, Millipore).

In analytical runs 0.15–0.4 ml of a 1–2% aqueous solution was applied to the columns via a septum injector. For preparative work samples of 30–80 mg of carbohydrate were applied in 0.3–1.0 ml to the P-6 column, whereas 200–300 mg of carbohydrate were loaded in *ca.* 2 ml on the P-4 columns.

The carbohydrates were detected by their absorbance at 420 nm after an aliquot of the column effluent had reacted with orcinol–sulphuric acid reagent in an automated analysis system[2]. The detector was assembled from modular components: a peristaltic pump (Type PLG; Desaga, Heidelberg, G.F.R.), a 15-m glass reaction coil in a 95°C heating bath (Haake N2B), and a photometer with a recorder. Glass coils, fittings and acid-resistant pump tubings were obtained from Cenco or Technicon. In preparative runs the column effluent was passed through a refractive index detector (Types 51.78 and 51.88, Knauer).

Preparation of oligosaccharides

Malto-oligosaccharides were obtained by partial acid hydrolysis of amylose (potato type III, Sigma) under the following conditions: (A) 2 g of amylose were dissolved in 100 ml of boiling water and 100 ml of hot 0.5 *M* sulphuric acid were added. The resulting solution (0.25 *M* sulphuric acid) was kept boiling for exactly 13 min on a magnetic stirrer. The hydrolysate was cooled in an ice-bath and was immediately neutralized with solid barium carbonate, centrifuged and lyophilized. (B) Partial acid hydrolysis of amylose was carried out under the conditions described above with the exception that 100 ml of 0.25 *M* sulphuric acid were used (final sulphuric acid concentration 0.125 *M*).

Arabinoxylan from oat spelt (Roth, Karlsruhe, G.F.R.) was purified from contaminating α-glucan (*ca.* 16%) by successive treatment with α-amylase of *Bacillus subtilis* and amyloglucosidase of *Aspergillus niger* (Boehringer, Mannheim, G.F.R.). The glucan-free arabinoxylan was removed from the digest by precipitation with ethanol (3 volumes), washed twice and lyophilized. The purified arabinoxylan was suspended in warm water (*ca.* 60°C) and the soluble fraction was separated from insoluble arabinoxylan by centrifugation. The soluble arabinoxylan fraction contained 12% arabinose and 88% xylose and was used for the preparation of xylo-oligosaccharides. The purified arabinoxylan was partially hydrolysed in boiling 0.125 *M* sulphuric acid for 12 min. The procedure was the same as described for amylose under conditions B.

Branched polymaltotrioses were prepared by the action of pullulanase on pullulan. The incubation mixture (5.0 ml; 30°C) contained 500 mg of pullulan (Sigma) in 50 m*M* acetate buffer (pH 5.0) and 5 units of pullulanase from *Aerobacter aerogenes* (Boehringer). After incubation at 60 min the mixture was inactivated by heating, centrifuged and lyophilized.

Iodine staining of malto-oligosaccharides

Partial acid hydrolysates of amylose were prepared according to conditions A and B and were mixed in the ratio 1:1. A 30-mg amount of this mixture was fractionated on a column (210 × 1.8 cm I.D.) of Bio-Gel P-6. The column effluent was monitored with a refractometer and fractions of 2.6 ml were collected. The fractions were lyophilized and carbohydrates were dissolved in 200 μl of hot water. Iodine solution (100 μl, 0.01 *N*) was added for staining.

Other methods

Mono- and oligosaccharides obtained from the partial acid hydrolysis of arabinoxylan were identified as described[15]. The acid hydrolysate of arabinoxylan was also analysed by high-performance liquid chromatography (HPLC) on a column (90 × 0.9 cm I.D.; 85°C) packed with Aminex 50W-X4 (Ca^{2+}) (20–30 μm)[16]. The column was eluted with water and the effluent was monitored by an automated analysis system as described above.

RESULTS

Branched polymaltotrioses with a degree of polymerization ranging from 3 to 60 were separated on a Bio-Gel P-6 column within less than 20 h (Fig. 1). A good resolution is achieved within a molecular weight range of 180–10,000, which is due to a molecular weight difference of one trisaccharide unit. The oligomer series was prepared by enzymic hydrolysis of pullulan, which is a linear α-glucan produced by *Pullularia pullulans* and in which maltotriose and small amounts of maltotetraose units are linked by 1,6-glycosidic bonds[17]. The different polymaltotrioses were ob-

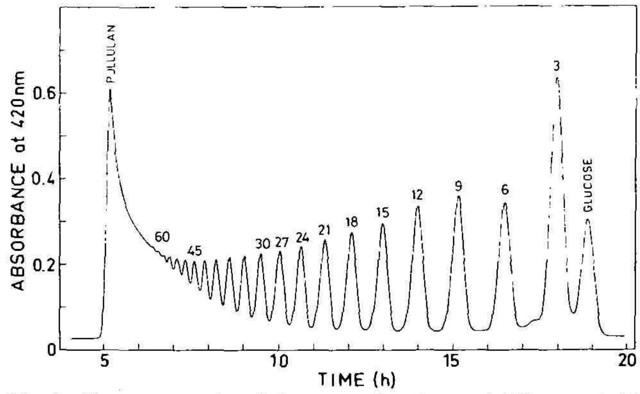


Fig. 1. Chromatography of glucose, maltotriose and different α-1,6-linked polymaltotrioses on Bio-Gel P-6. The column (gel bed, 197.6 × 1.8 cm I.D.) was eluted at 60°C with water at a flow-rate of 25 ml $\cdot$ h^{-1}. Sample size, 0.35 ml of a 2 % solution. Oligosaccharides were prepared as described in the text and glucose was added as a marker. The numbers over the peaks indicate the DP.

tained by the action of pullulanase from *Aerobacter aerogenes*, which randomly hydrolyses the α-1,6-glycosidic linkages in pullulan.

A plot of the negative logarithm of the distribution coefficient (K_{av}) *versus* the degree of polymerization of polymaltotrioses (Fig. 2) yields the expected linear relationship with an almost constant increment of $\Delta - \log K_{av} \approx 0.05$ going from one member in this series to the next higher member. The results of linear regression analysis of $-\log K_{av}$ *versus* DP are given in the legend of Fig. 2.

The well known reaction of amylose and its degradation products to produce a colour with iodine has been studied extensively, but not many exact data exist on the effect of DP on colour formation. In order to demonstrate the effect of the chain

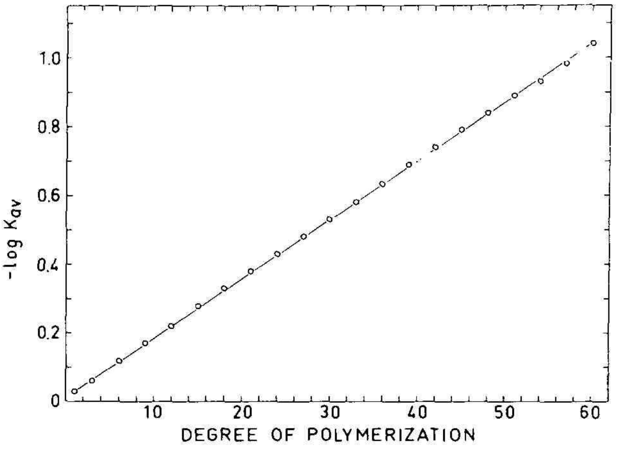


Fig. 2. Plot of $-\log K_{av}$ *versus* DP of α-1,6-linked polymaltotrioses. K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$, where V_e, V_0 and V_t are the solute elution volume, void volume and total volume, respectively, of the gel mass in the column. V_0 = 124.6 ml was determined by chromatography of dextran 500. Chromatographic conditions as in Fig. 1. Linear regression analysis: slope, 0.0171; intersection with the ordinate, 0.0179; correlation coefficient, 0.9999.

length of malto-oligosaccharides on the colour of the carbohydrate–iodine complex, two amylose hydrolysates with different oligosaccharide size distributions (prepared according to conditions A and B) were mixed and fractionated on Bio-Gel P-6. Owing to the small increase in molecular mass, retention data for malto-oligosaccharides were obtained only for the first twenty members of this homologous series and separation to the baseline was incomplete using this column. From the resulting elution profile the K_{av} values were calculated. Using the first twenty members of the oligosaccharide series, linear regression analysis was performed. The linear relationship made it possible to calculate DP > 20 in this incompletely separated malto-oligosaccharide series from the elution volume. After staining the separated malto-oligosaccharides with iodine, the colour of the carbohydrate–iodine complex was correlated with the appropriate DP (Table I).

TABLE I

EFFECT OF CHAIN LENGTH OF MALTO-OLIGOSACCHARIDES ON THE COLOUR OF THE CARBOHYDRATE–IODINE COMPLEX

Degree of polymerization (DP)	*Colour of carbohydrate–iodine complex*	*Wavelength of maximum absorbance, λ_{max} (nm)**
2–18	None	–
19–24	Brown	460 (23)
25–29	Red	505 (27)
30–38	Red-violet	515 (31)
39–46	Blue-violet	545 (41)
>47	Blue	562 (59)

* The wavelength was measured at the DP given in parentheses.

Fig. 3 illustrates the separation of different series of homologous oligosaccharides on two coupled Bio-Gel P-4 columns. The lower chromatogram shows the separation of an amylose hydrolysate. Optimal resolution was obtained up to a chain-length of approximately 25 glucose units. With Bio-Gel P-4 columns branched polymaltotrioses were completely separated up to DP 40 (upper part of Fig. 3). Useful elution profiles of both hydrolysates were also obtained in half the time (10 h) on increasing the flow-rate to 100 ml · h^{-1} (column pressure *ca.* 2.5 bar). In preparative runs samples of 200–300 mg were fractionated on the coupled Bio-Gel P-4 columns and no loss in resolution was observed. Refractive index detection was used for this purpose. When the refractometer response measured as peak height is plotted as a function of carbohydrate concentration the resulting calibration graph shows that glucose and malto-oligosaccharides fall on the same line (data not shown).

Chromatography on Bio-Gel P-4 was used for the large-scale preparation of linear xylo-oligosaccharides. An elution profile of a partial acid hydrolysate of arabinoxylan is shown in Fig. 4. The arabinoxylan used in this study is characterized by the presence of arabinofuranose side-chains, which are extremely susceptible to acid hydrolysis. By mild acid treatment arabinose was completely removed from the higher oligosaccharides (X_4–X_n). As can be deduced from the HPLC results shown in the inset of Fig. 4, the di- and trisaccharide fractions contained mainly linear xylo-

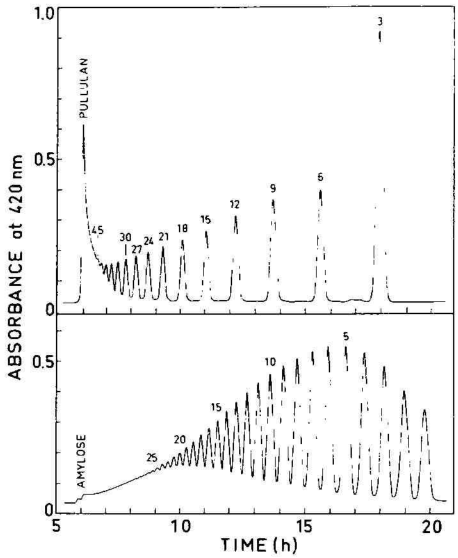


Fig. 3. Chromatography of oligosaccharides on Bio-Gel P-4. The upper figure shows the separation of α-1,6-linked polymaltotrioses from an enzymic digest of pullulan. The lower figure shows the elution profile of malto-oligosaccharides, which were obtained by partial acid hydrolysis of amylose according to conditions A as described under Experimental. Gel bed, 201 × 2.54 cm I.D.; column temperature, 60°C; eluent, water; flow-rate, 55 ml · h^{-1}. The numbers over the peaks indicate the DP.

oligosaccharides as well as minor amounts of arabinose-containing oligosaccharides. Confirmation that all isolated oligomers (X_4–X_n) are β-1,4-linked was obtained by degradation studies with highly purified β-xylosidase and xylanase preparations. UV spectra and enzymic degradation studies of the X_n peak revealed that this fraction contained lignin and small amounts of higher xylo-oligosaccharides.

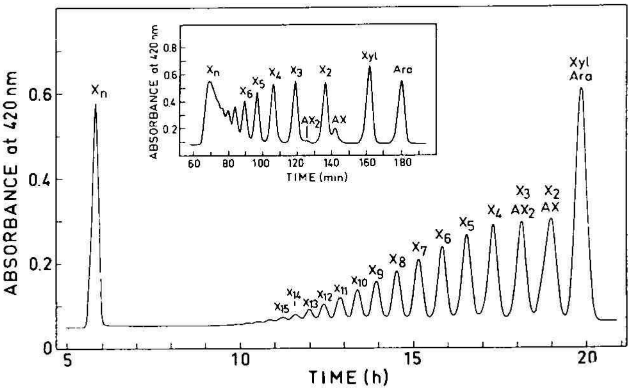


Fig. 4. Preparation of linear xylodextrins by partial acid hydrolysis of arabinoxylan and gel chromatography of the oligosaccharides on Bio-Gel P-4. The inset shows HPLC results for the same sample. Acid hydrolysis of arabinoxylan and peak identification are described under Experimental. Chromatographic conditions as in Fig. 3. Peaks: Xyl = xylose; Ara = arabinose; X_2, X_3, X_4, etc. = denote xylobiose, xylotriose, xylotetraose, etc.; AX = L-arabino-D-xylose; AX_2 = arabinoxylobiose; X_n = oligosaccharides with DP > 50.

DISCUSSION

We have performed oligosaccharide separations within a molecular weight range of 180–10,000 using 40 ± 4- and 47 ± 4-μm polyacrylamide gel particles with an appropriate pore size as column packings. The packing materials were obtained from commercial gels by an elutriation procedure.

Excellent separations of acetylated oligosaccharides containing up to 35 sugar residues have been achieved by reversed-phase liquid chromatography on bonded-phase silica[18] using an acetonitrile gradient (10–70% acetonitrile) as the eluent. However, the solubility of higher oligosaccharides in aqueous acetonitrile decreases as the DP increases and polysaccharides tend to bind irreversibly to the bonded phase packings. HPLC on cation-exchange resins in the silver[19] or calcium form[16,20] with water as eluent has been used successfully for the separation of mono- and oligosaccharides up to DP 15. For preparative purposes these HPLC techniques are not very useful, because with large sample amounts the resolution begins to deteriorate significantly, and hence long column lifetimes cannot be expected. Moreover, the cost of preparative columns packed with a high-performance support will limit this technique to analytical use.

Although major efforts were made to advance HPLC for the analysis of oligosaccharides, gel chromatography has remained an important technique for separating and isolating large oligosaccharides. The advantage of the system described in this paper over the many existing methods of analysis of oligosaccharide mixtures is the capability of separating oligomers up to DP 60, and as water is used as eluent at 60°C no solubility problems occur even with large oligomers. Moreover, the columns can be used for both analytical and preparative runs. Further advantages of the Bio-Gel techniques have been discussed earlier[1,2]. There is a disadvantage, however, in using the soft Bio-Gel P-6, as it tends to collapse at too high column pressures and high flow-rates should therefore be avoided.

The Bio-Gel P-4 columns have been used repeatedly for over 1 year and no deterioration of column performance was observed. Further particle size reduction of the relatively rigid Bio-Gel P-4 seems possible and would increase the column efficiency considerably.

ACKNOWLEDGEMENT

Michael John is much indebted to the Gesellschaft für Biotechnologische Forschung mbH, Braunschweig-Stöckheim, G.F.R., for financial support.

REFERENCES

1 M. John, G. Trénel and H. Dellweg, *J. Chromatogr.*, 42 (1969) 476.
2 M. John and H. Dellweg, in E. S. Perry and C. J. van Oss (Editors), *Separation and Purification Methods*, Vol. 2, Marcel Dekker, New York, 1973, p. 231.
3 B. S. Enevoldsen and F. Schmidt, *Proceedings of the 14th Congress of the European Brewery Convention*, Elsevier, Amsterdam, 1973, p. 135.
4 N. K. Sabbagh and I. S. Fagerson, *J. Chromatogr.*, 86 (1973) 184.
5 K. Kainuma, A. Nogami and C. Mercier, *J. Chromatogr.*, 121 (1976) 361.
6 J. Havlicek and O. Samuelson, *Carbohydr. Res.*, 22 (1972) 307.

7 A. Heyraud and M. Rinaudo, *J. Liquid Chromatogr.*, 4, Suppl. 2 (1981) 175.
8 F. Schmidt and B. S. Enevoldsen, *Carbohydr. Res.*, 61 (1978) 197.
9 F. E. A. van Houdenhoven, P. J. G. M. DeWit and J. Visser, *Carbohydr. Res.*, 34 (1974) 233
10 J.-F. Thibault, *J. Chromatogr.*, 194 (1980) 315.
11 A. Kobata, K. Yamashita and Y. Tachibana, *Methods Enzymol.*, 50 (1978) 216.
12 M. Natowicz and J. U. Baenziger, *Anal. Biochem.*, 105 (1980) 159.
13 J. C. Giddings, *Dynamics of Chromatography*, Marcel Dekker, New York, 1965.
14 A. Schwartz and B. A. Zabin, *Anal. Biochem.*, 14 (1966) 321.
15 M. John, B. Schmidt and J. Schmidt, *Can. J. Biochem.*, 57 (1979) 125.
16 H. D. Scobell, K. M. Brobst and E. M. Steele, *Cereal Chem.*, 54 (1977) 905.
17 B. J. Catley, J. F. Robyt and W. J. Whelan, *Biochem. J.*, 100 (1966) 5P.
18 G. B. Wells and R. L. Lester, *Anal. Biochem.*, 97 (1979) 184.
19 H. D. Scobell and K. M. Brobst, *J. Chromatogr.*, 212 (1981) 51.
20 J. Schmidt, M. John and C. Wandrey, *J. Chromatogr.*, 213 (1981) 151.

Journal of Chromatography, 247 (1982) 289–295
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,056

DETERMINATION OF FORMALDEHYDE IN AIR BY GAS CHROMATOGRAPHY

T. DUMAS

Agriculture Canada, London Research Centre, University Sub Post Office, London, Ontario, N6A 5B7 (Canada)

(Received April 13th, 1982)

SUMMARY

Formaldehyde in air was analyzed by gas chromatography (GC) using instruments equipped with thermal conductivity, flame-ionization and photoionization detectors. A method of making formaldehyde standards in air, to calibrate the GC system, was tested. The rate of polymerization increase in air, with increase in relative humidity, was also determined. The application of the method to the determination of formaldehyde desorbing from urea–formaldehyde foam and particle board is discussed.

INTRODUCTION

Formaldehyde (HCHO) is widely used in many industrial and domestic processes such as adhesive manufacture, electroplating, fermentation, fireproofing agents, pigment manufacture, for cotton durable press, preservatives for wool, synthetic resins, mirrors, in the production of urea–formaldehyde foam, an anticoagulant for natural rubber and in agriculture for the control of insects in fresh vegetables. Formaldehyde in air polymerizes easily[1] to a linear polymer, $HO(CH_2O)_nH$, but low levels of formaldehyde could remain for some time. Because it may be a hazard to human health, a level of 2 ppm has been set by AGGIH[2] as the TLV–TWA for an 8-h exposure. To determine such low levels with accuracy and reliability, very sensitive methods are required.

Methods currently available rely on large volumes, complex apparatus and chemical reactions. The NIOSH[3,4] method requires a sample size of 6–25 l, a procedure involving several steps and elaborate apparatus. Methods using detector tubes require sample sizes of 300–1600 ml for analysis in the 35–0.5 ppm range. A recent gas chromatographic–mass spectrometric (GC–MS) method[5] determines formaldehyde at the parts per billion level by trapping a formaldehyde–air mixture. In this method cooling at −72°C is used for conditioning the trap and a temperature of 240°C is required to release formaldehyde from the trap. Elaborate apparatus is also necessary. As GC can provide direct methods for the determination of vapors, equipment and procedures for the on-site determination of formaldehyde in a variety of environments were investigated and methods of analysis developed.

0021-9673/82/0000-0000/$02.75

EXPERIMENTAL

For the direct determination of formaldehyde in air at parts per million levels, GC was selected. For these GC determinations, three instruments with three different detectors were used, but it was first necessary to develop methods for making precise standards of formaldehyde in air.

These standards were generated from formaldehyde decomposed by heat on a stainless-steel plate placed on a hot-plate. The stainless-steel plate was heated to 160°C and an inverted beaker placed on it so that small amounts of paraformaldehyde could be introduced and formaldehyde generated to produce a saturated atmosphere. The size of the beaker was 10–100 ml, depending on the amount of formaldehyde needed. The formaldehyde–air mixture was drawn from this space with a syringe and injected directly into a gas chromatograph or into flasks of various sizes for subsequent analysis. As formaldehyde is explosive in the range 7–73% by volume in air, precautions were taken to produce only small amounts of vapor on the hot-plate and to provide adequate protection against flying glass in the event that an explosion occurred. Another method for producing the vapors at a low concentration involved introducing 16–100 mg of paraformaldehyde powder into 12.7-l flasks and applying heat to the powder to release formaldehyde. The first gas chromatograph used for this part of the investigation was a Bendix Model 2300 with a flame-ionization detector (FID) and a nickel column (2 m × 3 mm I.D.) packed with Tenax GC (35–60 mesh). At 40°C and a nitrogen flow-rate of 20 ml/min the retention time of formaldehyde was 3.15 min. A sensitivity setting of $5 \cdot 10^{-12}$ was used. By using the FID the effect of time and concentration on formaldehyde vapor was tested by drawing formaldehyde–air samples of 1–5 ml from various flasks at time intervals ranging from 1 min to 60 days and then analyzed. Another factor affecting polymerization is the relative humidity (RH), and this was also tested using GC with an FID. Levels of 30–70 and 75% RH were obtained by keeping the flasks in a room with a controlled RH. The 10% RH air or nitrogen atmospheres were obtained by flushing the flasks for 1 h with the respective gases from compressed gas cylinders and the RH was determined by GC. Samples up to 5 ml were injected directly into the GC system. For larger samples the trapping method previously described by Dumas[6] was used. For the trapping of formaldehyde a tube (20 cm × 3 mm I.D.) filled with Chromosorb 101 (60–80 mesh) was found to be effective. The trap was maintained at 25°C. Using this method, 10-ml samples of formaldehyde–air mixture were analyzed by injection into a trap at 25°C and placing the trap in the GC system, where formaldehyde was released at 60°C.

A second gas chromatograph, a Bendix Model 2200 with a thermal conductivity detector (TCD), was also used with the same column to determine the air in the formaldehyde–air mixture. The conditions used with this chromatograph were the same as used previously except that helium was used as the carrier gas and the retention time of formaldehyde was 2.7 min. The results were calculated with a Hewlett-Packard Model 3380A integrator.

A third gas chromatograph with very high sensitivity and portable so that it could be used for on-site analysis was also tested. This was the Photovac Model 10A10 instrument, equipped with a photoionization detector and a 1.2 m × 3 mm I.D. PTFE column packed with Carbopack BHT. A column temperature of 25°C and a carrier gas flow-rate of 12 ml/min in air (less than 0.1 ppm of hydrocarbon impurities) was used. Under these conditions the retention time of formaldehyde was 3.6 min. Either a 100-mV recorder or a Hewlett-Packard 3380A integrator was used to register the signal.

The desorption of formaldehyde from materials containing derivatives of this compound were tested using the above methods. Desorbing vapors from urea–formaldehyde foam were determined from 2 l (20 g) of foam placed in a 6-l desiccator and from 160 g of particle board placed in a 600-ml flask. Kitagawa and Dräger glass detector tubes were used to determine the formaldehyde content of the standards used for calibration. In a published GC method[5], various concentrations of formaldehyde in water were used as standards. To test such standards, a 37% formaldehyde solution stabilized with methanol was used to prepare dilute mixtures with water at concentrations down to 0.03%. These mixtures were tested by titration[7] to verify the actual concentration of formaldehyde.

RESULTS AND DISCUSSION

The possibility of preparing standards with low levels of formaldehyde in water was tested and the results in Table I show the actual formaldehyde contents determined by titration. The results for up to 100-fold dilution were close to the calculated amounts. For dilutions greater than 100-fold, the determined amounts were higher, being as high as 0.048% at 1000-fold dilution when the calculated amount was 0.03%. This interference makes the use of these dilute mixtures unreliable as standards.

TABLE I

FORMALDEHYDE CONCENTRATION IN SOLUTION DETERMINED BY TITRATION

Formaldehyde in solution (%)	
Calculated	*Determined*
37*	36.1
0.72**	0.77
0.37**	0.38
0.072**	0.082
0.037**	0.048
0.037**	0.042
0.037**	0.045
0.0185**	0.024

* 37% Formaldehyde solution stabilized with 13% of methanol.

** Dilutions with distilled water.

The instability of formaldehyde vapor makes it difficult to prepare an exact standard; however, at low levels, in air at levels of 120 ppm or less, the changes in concentration after 48 h is not detectable and this allows a formaldehyde–air mixture to be used as a standard for GC calibration.

Because paraformaldehyde decomposes at temperatures above 160°C to re-

TABLE II

DETERMINATION OF FORMALDEHYDE AT VARIOUS TIME INTERVALS AND RELATIVE HIMIDITIES USING DIFFERENT DETECTORS

Detector	*Flask volume*	*Sampling time*	*PF (mg)*	*Formaldehyde applied*	*Sample size*	*Formaldehyde integrator counts*	*Air integrator counts*	*Formaldehyde (ng/ml)* §	*RH (%)*	*HCHO (ppm)*
TCD	100 ml	1 min	100	100 μl	100 μl	42,000	82,686	–	–	–
TCD	100 ml	5 min	100	100 μl	100 μl	1300	134,000	–	–	–
TCD	100 ml	1 min	–	100 μl**	100 μl	–	133,500	–	–	–
FID	12.7 l	60 days	–	100 ml	1 ml	600	–	150***	33	–
FID	12.7 l	1 day	16*	–	5 ml	1400	–	70	70	50
FID	12.7 l	3 days	16*	–	5 ml	1100	–	55	70	–
FID	12.7 l	1 day	100*	–	5 ml	4500	–	225	75	162
FID	12.7 l	3 days	100*	–	5 ml	3600	–	180	75	–
FID	150 ml	60 days	–	5 ml	5 ml	2790	–	140	10	–
FID	150 ml	60 days	–	5 ml	5 ml	2200	–	110	30	–
FID	150 ml	60 days	–	5 ml	5 ml	1530	–	76	70	54
FID	100 ml	1 min	10	–	50 μl	12,000	–	3 μg/50 μl	–	–
Kitagawa	12.7 l	3 days	–	100 ml	100 ml	–	–	–	75	120

* Paraformaldehyde (PF) decomposed by heat into the 12.7-l flask, generating formaldehyde.

** Air only.

*** This amount was calculated based on Kitagawa reading of 120 ppm.

§ The amounts of formaldehyde were calculated based on the Kitagawa reading of the standard formaldehyde-air mixture.

lease formaldehyde, this procedure proved a convenient means of producing a mixture of formaldehyde in air. Table II shows the effect of various conditions on formaldehyde concentration in air. On drawing 100 μl of formaldehyde–air mixture into a syringe, after the first minute integrator counts of 82,686 for air and 42,000 for formaldehyde were obtained. On holding for 5 min the 100 μl of formaldehyde–air mixture in the syringe before injection, the counts were 134,000 for air and much lower (1300) for formaldehyde. The same amount of 100 μl of air alone showed 134,000 counts. This demonstrates that saturated formaldehyde in air could not be obtained because of its instability. By generating high levels of formaldehyde on the shot surface from paraformaldehyde and injecting 100 ml from the trapped vapor under the beaker into a 12.7-l flask, a standard was obtained. On sampling this standard after 6 days, 600 counts were obtained when 1 ml was injected into the gas chromatograph. On testing this flask with the Kitagawa glass detector tubes 120 ppm of formaldehyde was found, which corresponds to 150 ng/ml. Therefore, 1 ml containing 150 ng gave 600 counts on the integrator. Using this relationship, counts were converted into amounts of formaldehyde. The results for two 12.7-l flasks, containing 16 mg of paraformaldehyde in one and 100 mg in another, are shown in Table II. The paraformaldehyde was decomposed by heat, producing formaldehyde. The formaldehyde level in the flask with 16 mg of paraformaldehyde added at 70% RH, was 70 ng/ml after 1 day, and 55 ng/ml after 3 days. For the flask with 100 mg of paraformaldehyde added, at 75% RH the values were 225 ng/ml and 13.5 ng/ml after 1 and 3 days, respectively. This shows a higher loss at the higher concentration of formaldehyde.

The formaldehyde results for the 150-ml flasks at 10, 30 and 70% RH after 60 days showed that the remaining amounts of formaldehyde were becoming closer together, 140, 110 and 80 ng/ml. This indicates that at a low concentration and after a long time the rate of polymerization is very low, and the effect of RH is reduced.

The results in Fig. 1 show the effect of relative humidity and of formaldehyde concentration on the rate of polymerization as a function of time. The conversion was higher at the higher RH. This increases the rate of polymerization, especially in the first few minutes when the formaldehyde concentration is higher. For the 500 μg/150 ml (3333 ng/ml) of formaldehyde flask applied in a 10% RH atmosphere, after 15 min the concentration had fallen to 3000 ng/ml, where as at 70% RH it was much lower,

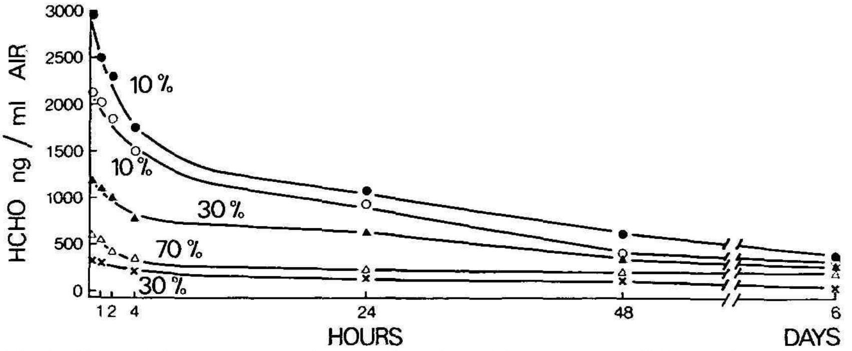


Fig. 1. Formaldehyde concentration as a function of time. ●,▲,△, 500 μg of formaldehyde applied in a 150-ml flask with air; ○, 500 μg of formaldehyde applied in a 150-ml flask with nitrogen; ×, 100 μg of formaldehyde applied in a 150-ml flask with air.

600 ng/ml. After 6 days the amounts of formaldehyde were much lower and closer together for all relative humidities with the same initial formaldehyde concentration. Comparing the polymerization at 30% RH, for initial applied amounts of formal-

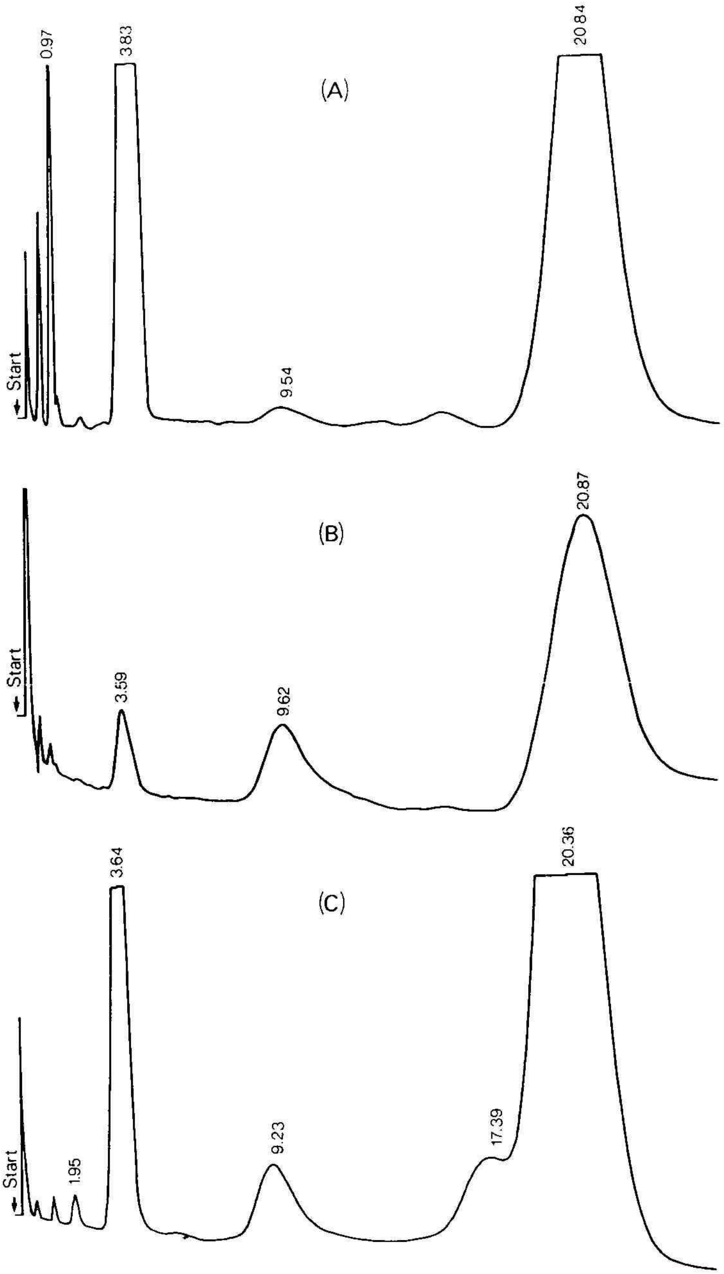


Fig. 2. Determination of formaldehyde using gas chromatograph with a photoionization detector. (A) 100-μl sample from particle board stored in a 600-ml flask; (B) 500-μl sample from formaldehyde standard in a 12.7-l flask; (C) 100-μl sample from urea–formaldehyde foam stored in a 6-l desiccator.

dehyde of 500 and 100 μg in the 150-ml flasks, after 6 days both were closer together in formaldehyde concentration. This indicates a continuous decrease in polymerization rate. Also, the polymerization rate was shown to be a function of concentration, time and relative humidity.

The desorption of formaldehyde from urea–formaldehyde foam was also tested, by placing samples in a 6-l desiccator for 6 months. Samples of 100 μl were drawn by a syringe and injected into the Photovac gas chromatograph for analysis. Fig. 2C shows the results for a 100-μl air sample from a 6-l desiccator containing 2 l (20 g) of urea–formaldehyde foam left for 6 months. The formaldehyde peak was at 3.64 min and there were also three other significant peaks, at 9.23, 17.39 and 20.36 min, which at present have been identified. The formaldehyde concentration for this sample was 120 ppm. When no interfering components with retention times close to 3.64 min are present in the air, a 1-ml sample could be injected into the gas chromatograph and smaller amounts of formaldehyde could be determined. The sample in Fig. 2B, generated from paraformaldehyde in the 12.6-l flask, produced the same two peaks at retention times of 3.59 and 20.87 min, which indicates that the peak at 20.36 min is due to formaldehyde and not to other components. In Fig. 2A, the same two peaks were also present for desorbed vapors from 160 g of particle board stored for 6 months in a 600-ml flask.

When formaldehyde–air standards of 55 ppm were analyzed using the FID, a 1-ml sample size produced a response of 286 counts on the integrator, but with the photoionization detector only 0.03 ml was required for a response of 285 counts. The photoionization detector has a higher sensitivity than the FID, but further studies, using the concentration method previously described by Dumas[6], which allows the analysis of larger samples, should be conducted to increase the sensitivity further.

ACKNOWLEDGEMENT

The author thanks Dr. E. J. Bond and Dr. H. V. Morley for helpful suggestions in preparing the manuscript.

REFERENCES

1 C. R. Noller, *Textbook of Organic Chemistry*, W. B. Saunders Co., Philadelphia, PA and London, 1957, p. 177.

2 *Threshold Limit Values for Chemical Substances and Physical Agents in the Workroom Environment*, American Conference of Government Industrial Hygienists, Cincinnati, OH, 1980.

3 *Formaldehyde in Air, Analytical Method No. P & CAM, 235*, U.S. Department of Health Education and Welfare, National Institute for Occupational Safety and Health, Cincinnati, OH, 1976.

4 *Formaldehyde Derivative Method No. S327*, U.S. Department of Health Education and Welfare, National Institute for Occupational Safety and Health, Cincinnati, OH, 1977.

5 Y. Yokouchi, T. Fujii, Y. Ambe and K. Fuwa, *J. Chromatogr.*, 180 (1979) 133.

6 T. Dumas, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 5.

7 J. F. Walker, *Formaldehyde*, ACS Monograph Series, Rheinold, New York, 1953, p. 380.

Journal of Chromatography, 247 (1982) 297–306
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,038

DETERMINATION OF FORMALDEHYDE AND OTHER ALDEHYDES IN AUTOMOBILE EXHAUST WITH AN IMPROVED 2,4-DINITROPHENYLHYDRAZINE METHOD

FRANK LIPARI and STEPHEN J. SWARIN*
Analytical Chemistry Department, General Motors Research Laboratories, Warren, MI 48090 (U.S.A.)
(Received May 17th, 1982)

SUMMARY

An improved, simple and rapid method for the determination of formaldehyde and other aldehydes in automobile exhaust was developed. Sample collection and derivatization are performed directly in a midget impinger containing an acetonitrile solution of 2,4-dinitrophenylhydrazine and catalyst. This scheme eliminates the time-consuming and lengthy recovery steps required in other procedures and allows direct injection of an aliquot of the sample into a high-performance liquid chromatograph for analysis. Detection limits for formaldehyde, acetaldehyde, acrolein and benzaldehyde are 20, 10, 5 and 4 ppb*, respectively, for a 20-l exhaust sample. The analysis time is as short as 10 min if only formaldehyde and acetaldehyde are of interest. The technique was used to measure aldehyde emissions from ethanol-, gasoline- and diesel-fueled vehicles.

INTRODUCTION

Aldehydes are produced by many industrial processes and combustion sources, including automobile engines. Formaldehyde and many of the other aldehydes are known contributors to photochemical smog[1] and irritants of the skin, eyes and nasopharyngeal membranes[2]. Formaldehyde is postulated to react with ionic chloride compounds in the air to produce bischloromethyl ether[3,4], a suspected carcinogen. Studies conducted at the Chemical Industry Institute of Toxicology indicate an increased incidence of nasal cancer in rats exposed to high concentrations (15 ppm) of formaldehyde[5]. The current Occupational Safety and Health Administration (OSHA) 8-h time-weighted average permissible exposure limit for formaldehyde is 3 ppm[6]. Therefore, because of the environmental importance of these compounds, a sensitive and rapid technique for their determination in automobile exhaust is needed.

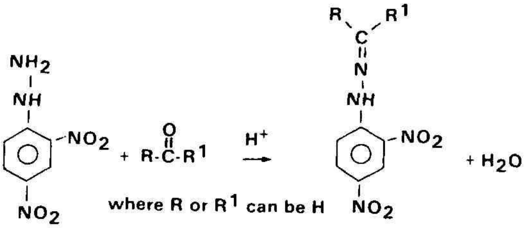
The two commonly used methods for the analysis of formaldehyde and other aldehydes in automobile exhaust are the 3-methyl-2-benzothiazolone (MBTH) and the 2,4-dinitrophenylhydrazine (DNPH) methods. The MBTH[7] method is a non-

---

* Throughout this article, the American billion (10^9) is meant.

selective colorimetric method for low-molecular-weight aliphatic aldehydes. This method measures total aldehydes in terms of their formaldehyde equivalents. The method is insensitive to higher molecular weight and unsaturated aldehydes (*e.g.*, acrolein and benzaldehyde) and recently was shown to be subject to a 25% negative interference from sulphur dioxide in automobile exhaust samples[8].

In the DNPH method, individual aldehydes and ketones react with an acidic solution of DNPH to form hydrazone derivatives as shown in eqn. 1:



(1)

These derivatives are removed from the aqueous solution by filtration, extraction, evaporation to dryness, and dilution in a solvent suitable for gas chromatographic (GC)[9–11] or high-performance liquid chromatographic (HPLC)[12–15] analysis. Although the method separates the aldehydes and ketones and quantitates them individually, the extensive sample preparation is time consuming, and sample recoveries for various aldehydes and ketones range from 60 to 85%.

This paper describes an improved method for the determination of formaldehyde and other aldehydes in which the sample collection and the derivatization reaction are performed directly in a midget impinger containing an acetonitrile solution of the DNPH reagent. This scheme eliminates the filtration, extraction and transfer steps and allows direct injection of the sample into the HPLC system. During the course of this work, Kuntz *et al.*[16] described a similar procedure. Our report presents further details of this procedure, including collection efficiency studies, breakthrough studies, improved separation of the aldehyde derivatives and defined detection limits. This technique was used to measure aldehyde emissions from ethanol-, gasoline- and diesel-fueled vehicles.

EXPERIMENTAL

Apparatus

All HPLC separations were performed on either a Varian Model 5000 equipped with a 254-nm detector or a Waters Model 244 liquid chromatograph equipped with a Model 440 UV detector. Ultraviolet detection with the Waters instrument was effected at either 254 or 365 nm. Solvent programs were generated via microprocessor control with the Varian instrument and with a Model 1612-B program control module (Munhall Co.) for the Waters instrument. Samples were injected onto the column in the Varian system with a Valco injector and on to the column in the Waters system with a Waters Model U6K injector. The sample sizes injected were 30 μl. Various commercially available reversed-phase (ODS) chromatographic columns were used to achieve the most efficient separations. These included a 15 cm × 4.6 mm I.D. Supelcosil LC-18 (Supelco), a 25 cm × 4.6 mm I.D. Ultrasphere-ODS (Beckman) and a 25 cm × 4.6 mm I.D. Zorbax-ODS column (DuPont).

Reagents

The mobile phase was prepared from HPLC-grade "distilled in glass" acetonitrile (Fisher Scientific) and deionized water. The 2,4-dinitrophenylhydrazine was obtained from both J. T. Baker and Aldrich. The DNPH was recrystallized from HPLC-grade methanol to reduce carbonyl impurities. All other chemicals and the aldehydes were of the best available grade from various suppliers.

Derivative preparation

The DNPH derivatives were prepared by reacting the corresponding aldehydes with saturated DNPH solution in 2 *N* hydrochloric acid. The DNPH derivatives were filtered, washed with water and 2 *N* hydrochloric acid, air dried and stored in closed vials. Purity was checked by melting-point determinations and by liquid chromatographic analysis. Some of the derivatives were recrystallized from absolute ethanol.

Preparation of solutions and standards

A 6.25 μmole/ml DNPH stock solution was prepared by addition of 125 mg of purified DNPH crystals to 100 ml of acetonitrile. Dilution of the stock solution with acetonitrile was used to prepare a 3.1 or a 1.6 μmole/ml DNPH absorbing solution. The catalyst (1 *N* perchloric acid) was added to the absorber solution just before use (1 drop per 5 ml of absorber).

Standard solutions of the aldehydes were prepared by injecting 5.0 μl of the aldehyde into a septum sealed vial containing 5.0 ml of acetonitrile. The concentrations were calculated from the densities of the aldehydes or, in the case of formaldehyde, from the concentration of the stock solution as determined by titration[17]. These solutions were used to generate known atmospheres of aldehydes. Standard derivative solutions were prepared by injecting 2.0–20 μl of the aldehyde solution into a septum-sealed vial containing the derivatizing reagent and also by weighing the solid hydrazones and dissolving them in acetonitrile.

Generation of standard aldehyde vapors

A Kin-Tek certified paraformaldehyde (α-polyoxymethylene) permeation tube together with a Kin-Tek Model 670 permeation tube system was used to generate known atmospheres of formaldehyde.

To generate known atmospheres of acetaldehyde, acrolein and benzaldehyde, the method described by Graham[18] for the generation of known atmospheres of isocyanates was adapted. The experimental apparatus shown in Fig. 1 was employed. A known amount of an aldehyde solution in acetonitrile (10–30 μl) was placed in impinger A and 10 or 20 ml of the absorber solution was placed in impinger B. Room air was then drawn through both impingers at rates varying from 0.5 to 1.5 l/min with a Bendix BDX-55-HD Super Sampler Pump (Bendix Corp., Lewisburg, WV, U.S.A.). The sampling time was varied so that a total volume of 20 l of air was drawn through the impingers. During this sampling time, impinger A was heated for 10–45 sec with a heat gun to ensure volatilization of the aldehydes. The aldehydes were

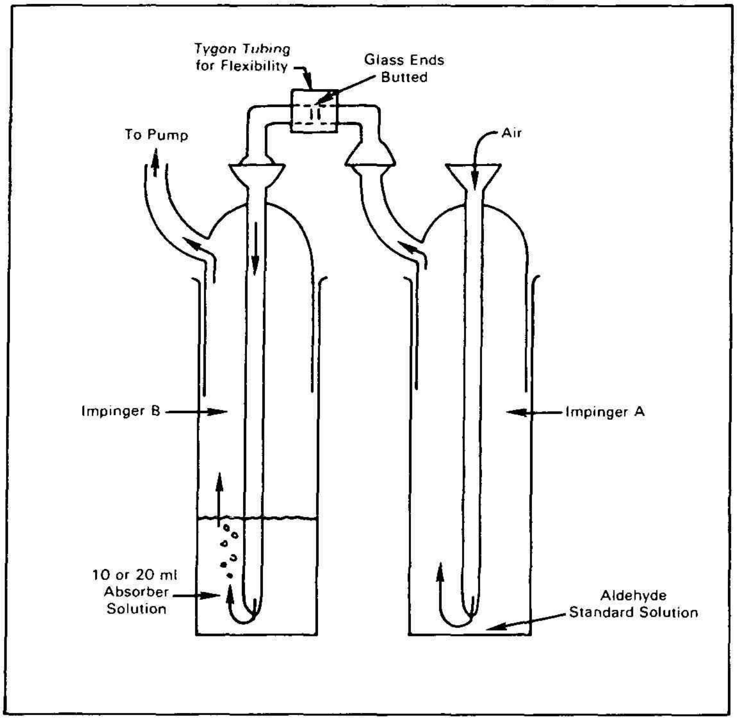


Fig. 1. Apparatus for generation of known aldehyde vapors.

swept out of impinger A and trapped in the absorber solution in impinger B. This technique was used for the recovery and precision studies described here.

Procedure for exhaust sampling

Aldehyde emissions from various vehicles were measured as the test cars were driven on a chassis dynamometer. One sample from each test phase was taken from a cold-start, 1-cycle 1972 Federal Test Procedure (FTP) and a hot-start 18-cycle 1972 FTP test. The raw exhaust was diluted with room air by a constant volume sampler (CVS). Exhaust samples taken for analysis were collected by bubbling the diluted exhaust from the CVS through two midget impingers (Ace Glass, Vineland, NJ, U.S.A.) connected in series. Each impinger contained 20 ml of the absorbing DNPH solution. The samples were drawn at a rate of 1.0 l/min by a Dynapump, and the sample volume was measured with a wet-test meter.

RESULTS AND DISCUSSION

Reaction kinetics of the derivatization reaction

For the derivatization reaction to be analytically useful, the reaction must proceed in an analogous manner in acetonitrile solvent to that in aqueous solution. A study of the effect of the acid catalyst (hydrochloric and perchloric acids) concentration on the reaction kinetics and derivative stability was undertaken. Known amounts (0.3–0.5 μmole) of formaldehyde, ethyl methyl ketone and benzaldehyde were injected into 4.0 ml of reagent (6.0 μmole) containing variable amounts of hydrochloric acid catalyst (1–4 drops of 1–12 N acid). The reaction mixture was analyzed by reversed-phase HPLC at various times after the reaction was initiated. The results indicate that the reactions proceed very fast (< 5 min) for the aldehydes at all hydrochloric acid concentrations and that the derivatives are stable for a least 6 h.

The reaction with hydrochloric acid produced a white precipitate, which was later identified by mass spectrometry as 2,4-dinitrophenylhydrazone hydrochloride. A similar study with perchloric acid gave identical reaction kinetics and produced no white precipitate, presumably because of the greater solubility of perchloric acid in acetonitrile. The derivatives were fairly stable (less than 10% decomposition after 24 h with perchloric acid) and subsequently perchloric acid was used in all work.

The optimum reaction conditions chosen were 1 drop (50 μl) of 1 N perchloric acid per 4.0 ml of reagent solution. The concentration of the reagent solution was generally $\leqslant 6.25$ μmole/ml, depending on the expected concentration of the aldehydes. At low aldehyde levels where blank impurities become a problem, the concentration of the reagent solution was decreased to 1.56 μmole/ml.

High-performance liquid chromatography

An investigation of the resolution obtainable for the DNPH derivatives with three commercially available columns was undertaken (see Experimental). Various mixtures of acetonitrile and water were used as the mobile phases.

The best separation of C_3 aldehyde and ketone derivatives, and indeed the best overall separation, was obtained with a 6-μm Zorbax-ODS column. As can be seen in Fig. 2, the acrolein, acetone and propionaldehyde derivatives are very well resolved whereas the ethyl methyl ketone and butyraldehyde derivatives are not. As acrolein is the more important pollutant, the separations were optimized for the C_3 species at the expense of the C_4 species. This separation scheme was used for most of the automobile exhaust work, as it offered complete resolution of the desired species in a reasonable time. However, with a slightly modified solvent program it is possible to separate even more components (Fig. 3).

Validation of the method

The collection efficiency using the proposed method for formaldehyde vapors was determined using a paraformaldehyde permeation tube. The permeation tube was found to deliver 2.40 μg/min of formaldehyde as determined by the MBTH method[7]. The concentration of formaldehyde was varied from 0.358 to 1.14 ppm by varying the dilution gas (helium) flow-rate of the system from 0.5 to 1.5 l/min. The total sampling time was kept constant at 20 min so that the gas volume sampled varied from 10 to 30 l while the total amount of formaldehyde generated was constant (48.0 μg). The collection efficiency for formaldehyde was 97.5 ± 1.0% (four determinations) using single midget impingers containing either 10 or 20 ml of reagent solution.

Atmospheres of other aldehydes were generated using the method and the apparatus described by Graham[18] (see Experimental). Standard 20-l atmospheres of acetaldehyde, acrolein and benzaldehyde with an average concentration varying from 0.10 to 0.50 ppm were generated using this technique. The overall collection efficiency

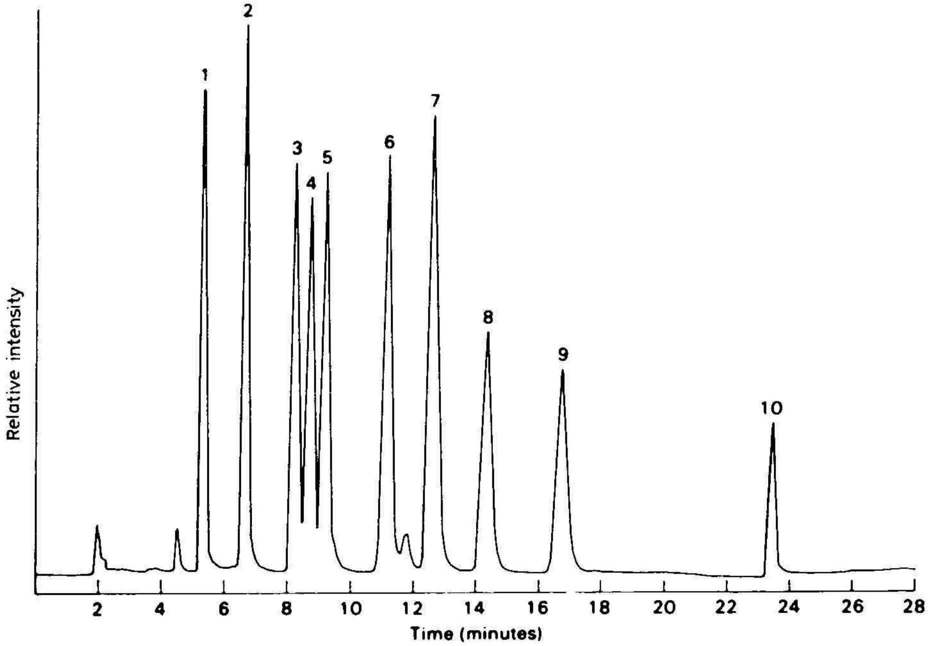


Fig. 2. Chromatogram of various DNPH derivatives separated on a Zorbax-ODS column: acetonitrile–water (65:35) at 1.0 ml/min for 20 min, then gradient to acetonitrile–water (80:20) over 10 min; UV detection at 365 nm. Peaks: 1 = formaldehyde; 2 = acetaldehyde; 3 = acrolein; 4 = acetone; 5 = propionaldehyde; 6 = crotonaldehyde; 7 = ethyl methyl ketone/butyraldehyde; 8 = benzaldehyde; 9 = isovaleraldehyde; 10 = hexanaldehyde.

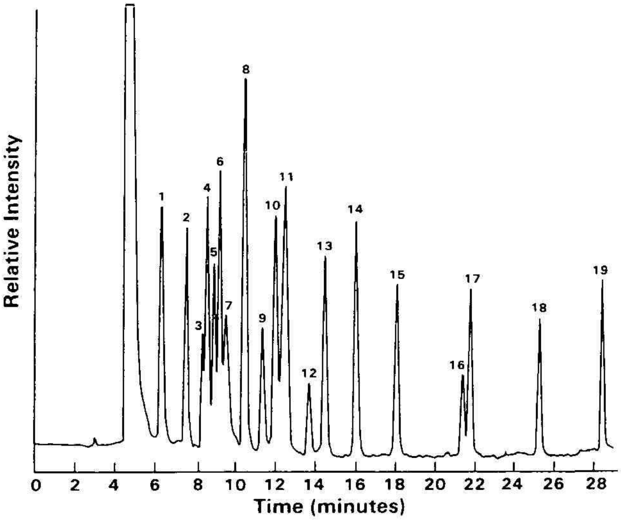


Fig. 3. Chromatogram of various DNPH derivatives separated on a Zorbax-ODS column: acetonitrile–water (67:33) at 0.7 ml/min for 8 min, then 1.0 ml/min and gradient to acetonitrile–water (90:10) over 17 min, then gradient to 100% acetonitrile over 3 min. Peaks: 1 = formaldehyde; 2 = acetaldehyde; 3 = furfural; 4 = acrolein; 5 = acetone; 6 = propionaldehyde; 7 = salicylaldehyde; 8 = crotonaldehyde; 9 = butyraldehyde; 10 = glyoxal; 11 = benzaldehyde; 12 = glutaraldehyde; 13 = valeraldehyde; 14 = *p*-tolualdehyde; 15 = hexanaldehyde; 16 = 3-heptanone; 17 = heptanaldehyde; 18 = octanaldehyde; 19 = nonanaldehyde.

for these aldehydes was 95.0 ± 4.0% (four determinations each). The precision probably reflects the reproducibility of using a syringe to dispense dilute aldehyde solutions into the generator impinger.

The collection efficiency of this method was compared with the aqueous DNPH procedure[15] by injecting 15-μl aliquots of dilute solutions of formaldehyde, acrolein, butyraldehyde and benzaldehyde (amounts ranged from 12 to 20 μg of each aldehyde) into 20 ml of acetonitrile–DNPH and 20 ml of 2 *N* hydrochloric acid–DNPH. The latter solution was extracted three times with pentane and the extracts were combined and evaporated to dryness and the residue was dissolved in 20 ml of acetonitrile. Analysis of the resulting acetonitrile solutions by HPLC allows a direct comparison of the collection efficiency of the two methods, as shown in Table I. The low recoveries and large relative standard deviations shown in this table are probably the result of variable losses during the extraction and evaporation steps required by the aqueous-based procedure. In any event, these collection efficiencies are inadequate for a good analytical method, and they show the superiority of the acetonitrile–DNPH procedure.

TABLE I

COLLECTION EFFICIENCY OF THE 2 *N* HYDROCHLORIC ACID–DNPH PROCEDURE

Efficiency relative to acetonitrile–DNPH procedure.

Aldehyde	*Collection efficiency (%)**
Formaldehyde	86 ± 10**
Acrolein	63 ± 10; 9 ± 4***
Butyraldehyde	80 ± 14
Benzaldehyde	102 ± 7

* Average of at least five determinations.

** Relative standard deviation.

*** The first value is for acrolein alone and the second for acrolein in a mixture with the other three aldehydes.

The detection limits for formaldehyde, acetaldehyde, acrolein and benzaldehyde are listed in Table II. These detection limits are given for UV detection at 365 nm where the DNPH derivatives have their maximum absorbance. Detection at 365 nm as opposed to 254 nm improves the signal-to-noise ratio by a factor of two for

TABLE II

DETECTION LIMITS FOR VARIOUS ALDEHYDES

Aldehyde	*Detection limit (ppb, v/v)**
Formaldehyde	20**
Acetaldehyde	10***
Acrolein	5
Benzaldehyde	4

* Based on a 20-l air sample, a 10-ml final impinger volume and a 30-μl injection.

** A blank peak equivalent to 10 ppb in the reagent based on a 20-l air sample.

*** A blank peak equivalent to 5 ppb in the reagent based on a 20-l air sample.

most of the aldehydes determined and, consequently, improves the detection limit by the same factor. Where no reagent blanks are found (acrolein and benzaldehyde), the detection limits were calculated based on an aldehyde concentration equivalent to twice the noise level. When reagent blank impurity peaks are present (formaldehyde and acetaldehyde), the detection limits are based on an aldehyde concentration equivalent to twice the blank impurity peak. The stock DNPH reagent (6.25 μmole/ml) contained 30–40 ppb of formaldehyde equivalents and 15–20 ppb of acetaldehyde equivalents, respectively. Most of the work utilized a 1:4 dilution of the stock reagent, thus reducing the blank impurities. Batch to batch variations in the DNPH reagent can lead to variance in reagent aldehyde impurities and thus affect the detection limits. The detection limit for formaldehyde and acetaldehyde would be about 2.0 ppb if the blank were zero. The detection limits can be improved by further purifying the reagent to remove formaldehyde and acetaldehyde impurities, by taking larger air samples or by increasing the injection volume.

A breakthrough study was carried out to determine the maximum amount of formaldehyde that can be effectively collected in a midget impinger and the point where the collection efficiency deviates from 100%. This study was conducted by bubbling formaldehyde vapor at the rate of 2.4 μg/min into a single midget impinger containing 20 ml (or 31 μmole) of DNPH reagent. The amount of formaldehyde delivered and, hence, the reagent to aldehyde molar ratio was varied by sampling for 20, 60 and 120 min at 1.0 l/min. The resulting molar ratios at these sampling times were 20:1, 6.6:1, and 3.3:1, respectively. Recoveries of 98 ± 4% were obtained at all three molar ratios tested. Even at a molar ratio of 1.65:1, recoveries in escess of 70% were obtained. Apparently, at a 3.3:1 molar ratio sufficient excess reagent exists to drive the reaction to completion. With 31 μmole of reagent in a midget impinger, 10 μmole of formaldehyde or other aldehyde species can be collected with nearly 100% collection efficiency.

Stability of the reagents and samples

The acetonitrile–DNPH solution is stable for at least 2 months when it is stored at 0°C. However, after the 1 *N* perchloric acid catalyst has been added, the solution will absorb aldehydes from the air and the catalyst is therefore added just before use.

This procedure was developed to give fast turnaround time in emission testing so most samples were analyzed within 1 h of collection. However, other applications of this technique may require longer time periods between the collection and analysis of samples. In our experience all of the aldehyde derivatives except acrolein are stable in acetonitrile solvent at room temperature for at least 2 weeks. The acrolein derivative decomposes in the presence of excess of DNPH and 1 *N* perchloric acid at room temperature so that only 30% of the original amount is left after 1 week. Refrigeration at 0°C retards this decomposition significantly so that 85% of the acrolein derivative is left after 1 week. Therefore, refrigeration of samples and standards at 0°C is recommended.

Application of the method to automobile exhaust samples

This method was used to determine aldehyde emissions from ethanol-, gasoline- and diesel-fueled vehicles. The data given in the tables are for diluted exhaust (*ca.* 10:1) from the CVS system (see Experimental).

Table III summarizes the results obtained for a prototype ethanol-fueled vehicle, a 1974 (no catalyst) gasoline-fueled vehicle and a 1981 (catalyst) gasoline-fueled vehicle. The cold-start and hot-start designations refer to 1972 Federal Test Procedure driving cycles. The major aldehyde emissions found for these vehicles were formaldehyde and acetaldehyde. These vehicle tests show the wide range of concentrations that can be encountered in emission testing and also show that the method developed is applicable to aldehyde emissions ranging from 0.04 to 28 ppm in diluted exhaust. Multiple HPLC analyses of the same sample yielded a reproducibility of ±4.2% (four determinations).

TABLE III

ALDEHYDE EMISSIONS FROM ETHANOL- AND GASOLINE-FUELED VEHICLES

Aldehyde	*Results (ppm, v/v) in diluted exhaust*					
	*Ethanol-fueled**		*1974 gasoline-fueled***		*1981 gasoline-fueled****	
	Cold start	*Hot start*	*Cold start*	*Hot start*	*Cold start*	*Hot start*
Formaldehyde	7.31	4.28	2.42	2.54	0.36	0.04
Acetaldehyde	20.30	10.10	0.67	0.59	0.08	ND
Acrolein	ND	ND	0.27	0.24	0.02	ND
Butyraldehyde	ND	ND	0.03	0.03	0.01	ND
Benzaldehyde	ND	ND	0.10	0.10	0.03	ND
Total	27.61	14.38	3.49	3.50	0.50	0.04

* Prototype 100% ethanol-fueled vehicle.

** No catalyst.

*** Computer command control emission system.

§ ND denotes not detected (< 0.01 ppm).

TABLE IV

ALDEHYDE EMISSIONS FROM DIESEL-FUELED VEHICLES

Aldehyde	*Results (ppm, v/v) in diluted exhaust*			
	*1978 vehicle**		*1980 vehicle***	
	Cold start	*Hot start*	*Cold start*	*Hot start*
Formaldehyde	0.57	0.47	0.70	0.49
Acetaldehyde	0.13	0.09	0.18	0.13
Acrolein	0.09	0.09	0.08	0.05
Propionaldehyde	0.06	0.06	0.05	0.03
Crotonaldehyde	0.01	0.01	0.01	0.01
Butyraldehyde	0.01	0.01	0.06	0.07
Benzaldehyde	0.01	0.01	0.02	0.02
Tolualdehydes	ND***	ND	ND	ND
Total	0.88	0 74	1.10	0.80

* 1978 production vehicle with a 5.7-l, V-8 diesel engine without exhaust gas recirculation.

** 1980 production vehicle with a 5.7-l, V-8 diesel engine with exhaust gas recirculation.

*** ND denotes not detected (<0.01 ppm).

Table IV shows the results obtained for two diesel-fueled automobiles. These data demonstrate the number of aldehydes that can be determined in a single test and the effect of engine configuration on individual aldehyde emissions.

The chromatographic analysis scheme shown in Fig. 2 was used for these tests. With these conditions, on analysis time of about 45 min per sample (including column equilibration) was required. However, when only a few aldehydes are important or expected, the analysis time can be reduced to about 10 min per sample.

ACKNOWLEDGEMENTS

We appreciate the assistance of S. G. Anderson and K. G. Kennedy who operated the emission facility, and G. N. Nebel who performed the MBTH analyses.

REFERENCES

1 R. A. Bailey, H. M. Clark, J. P. Ferris, S. Krause and R. L. Strong, in *Chemistry of the Environment*, Academic Press, New York, 1978, p. 268.
2 M. Sittig, in *Pollution Detection and Monitoring Handbook*, Noyes Data Corp., Park Ridge, NJ, 1974.
3 C. C. Yao and G. C. Miller, *Publication No. 79-118*, Department of Health Education and Welfare, National Institute for Occupational Safety and Health, Cincinnati, OH, 1969, p. 73.
4 W. S. Klim, C. L. Geraci and R. E. Kupel, *Amer. Ind. Hyg. Ass. J.*, 41 (1980) 334.
5 P. Gunby, *J. Amer. Med. Ass.*, 243 (1980) 1697.
6 *Title 29, Code of Federal Regulations*, Section 1910.1001, Table 2, U.S. Government Printing Office, Washington, DC.
7 M. Katz, *Methods of Air Sampling and Analysis*, APHA Intersociety Committee, Washington, DC, 2nd ed., 1977.
8 S. H. Cadle, G. N. Nebel and R. L. Williams, *SAE Trans.*, 88 (1980) 2381.
9 R. A. Smith and I. Drummond, *Analyst (London)*, 104 (1979) 875.
10 *Procedures for Determining Exhaust Carbonyls as 2,4-Dinitrophenylhydrazones; Final Report in CY 1968 under GRC ZPRZC Project No. CAPE-11-68, Fuels Combustion Research Project*, Bartlesville Petroleum Research Center, Bureau of Mines, Atlanta, GA, 1969.
11 L. J. Papa and L. P. Turner, *J. Chromatogr. Sci.*, 10 (1972) 774.
12 S. Selim, *J. Chromatogr.*, 136 (1977) 271.
13 K. Kuwata, M. Uebori and Y. Yamasaki, *J. Chromatogr. Sci.*, 17 (1979) 264.
14 L. J. Papa and L. P. Turner, *J. Chromatogr. Sci.*, 10 (1972) 747.
15 G. Creech, R. T. Johnson and J. O. Stoffer, *J. Chromatogr. Sci.*, 20 (1982) 67.
16 R. Kuntz, W. Lonneman, G. Namie and L. A. Hull, *Anal. Lett.*, A16 (1980) 1409.
17 *Reagent Chemicals*, American Chemical Society, Washington, DC, 1974, p. 274.
18 J. D. Graham, *J. Chromatogr. Sci.*, 18 (1980) 384.

Journal of Chromatography, 247 (1982) 307–314
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,062

RAPID AND RELIABLE METHOD FOR THE ANALYSIS OF NUCLEOTIDE POOLS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. MARTINEZ-VALDEZ
Department of Biochemistry, School of Medicine, National University of Mexico, 04510 Mexico City (Mexico)
and
R. M. KOTHARI, H. V. HERSHEY and M. W. TAYLOR*
Department of Biology, Indiana University, Bloomington, IN 47405 (U.S.A.)

(Received May 10th, 1982)

SUMMARY

A rapid and reliable protocol for the simultaneous separation of ribo-, deoxyribo- and cyclic nucleotides has been developed using high-performance liquid chromatography on a C_{18} μBondapak column and isocratic elution with ammonium phosphate buffer (0.2 *M*, pH 5.1). Resolution of deoxyribonucleotides has been confirmed by performing resolution before and after periodate oxidation. The general order of elution is ribonucleotides, deoxyribonucleotides and cyclic nucleotides. While periodate oxidation improved the clarity of separation of deoxyribonucleotides by eliminating ribonucleotides, incorporation of methanol in the eluent shortened the retention time of the cyclic nucleotides. The application of this method to a complex biological system is reported.

INTRODUCTION

We have recently reported[1] a rapid and sensitive method for separating ribonucleotides on a C_{18} reversed-phase column using isocratic elution with ammonium phosphate buffer. The elution profiles were dependent on both pH and ionic strength of the buffer. To our knowledge, none of the existing methods affords simultaneous detection of total ribo-, deoxyribo- and cyclic nucleotides using isocratic elution conditions. Some protocols provide for the separation of pyrimidine nucleotides alone[2], purine nucleotides alone[3] and exclusive separation of either ribonucleotides[4] or deoxyribonucleotides[5]. Using an isocratic elution buffer and a reversed-phase column, we now report the resolution of the deoxyribonucleotides and cyclic ribonucleotides from each other and from ribonucleotides. The deoxyribonucleotides and cyclic ribonucleotides elute, in general, later than the corresponding ribonucleotides and, depending on the analysis desired, their elution could be expedited using the same buffer fortified with 10% (v/v) methanol. Combining periodate treatment to

0021-9673/82/0000–0000/$02.75

remove ribonucleotides, deoxyribonucleotides can be easily distinguished from the ribonucleotides in biological samples. This protocol has been applied in analyzing the ribonucleotide, deoxyribonucleotide and cyclic nucleotide pool from HeLa cells and for the identification of major purine and pyrimidine species.

EXPERIMENTAL

Apparatus

For high-performance liquid chromatography (HPLC), a Model U6K sample injector, M45 solvent delivery system, either a Model 450 variable-wavelength detector or a Model 440 fixed-wavelength detector (Waters Assoc., Milford, MA, U.S.A.) and an Omniscribe automatic recorder (Houston Instruments, Austin, TX, U.S.A.) or a Hewlett-Packard Model 3390A integrator were used. A pre-packed reversed-phase column (30 × 0.4 cm I.D.; particle size 10 μm) containing an octadecyl (C_{18}) chemically bonded stationary phase (Waters Assoc.) was utilized. A pre-column (5 × 0.4 cm I.D.) also packed with C_{18} reversed-phase material was used to protect the main column.

Chemicals

Ribo-, deoxyribo- and cyclic nucleotides were obtained from Sigma (St. Louis, MO, U.S.A.). A 10 m*M* stock solution of each nucleotide was prepared in distilled water, stored at −70°C and diluted before use. All other chemicals used were of analytical-reagent grade. Methanol of HPLC grade was obtained from Waters Assoc. Ammonium phosphate buffer (0.2 *M*, pH 5.1) was freshly prepared in doubly distilled water, pre-filtered through a 0.2-μm Millipore filter and degassed extensively before use.

Periodate oxidation

In principle, the method of Neu and Heppel[6] was followed, with minor modifications. Proportional volumes of ribonucleotides and deoxyribonucleotides were mixed to a final concentration of 1.6 μ*M*. Aliquots of 1 ml of this mixture were treated at ambient temperature for 10 min with 80 μl of 1 *M* sodium periodate, after which 10 μl of 4 *M* cyclohexylamine were added and the resultant mixture was incubated at 45°C for 90 min. After incubation, 10 μl of 0.5 *M* rhamnose were added to remove excess of iodate ions. A 10-μl aliquot was chromatographed from the periodate-oxidized samples.

Silica cartridge chromatography

In order to avoid confusion among ribonucleotides (cytosine and uracil plus thymine series) and free bases which elute with a very close retention time with that of periodate and cyclohexylamine, samples were fractionated according to Lothrop and Uziel[7] through silica cartridge (Waters Assoc.) after periodate oxidation. The resultant free deoxyribonucleotides were then chromatographed on a C_{18} column as for the purine series.

Preparation of HeLa cell extracts

HeLa cell cultures were grown in Hank's MEM containing 10% newborn calf serum at 37°C until confluent. The medium was discarded and the cells were washed three times with chilled PBS and extracted in 6 ml of 0.66 *M* formic acid at 4°C for 15 min. The extract was centrifuged (4°C, 3000 r.p.m., 10 min), the supernatant passed through a C_{18} cartridge (Waters Assoc.) at a flow-rate of 1 ml/min, the cartridge washed with 15 ml of distilled water and the pooled sample was lyophilized. The dry material was extracted in 1 ml of distilled water and filtered through a 0.2-μm Millipore filter before chromatography.

Chromatographic conditions

A μBondapak C_{18} column was used in these studies. The column was washed daily with doubly distilled water, followed by methanol–water (30:70), and preserved in the latter between use. Before use, it was equilibrated with water and ammonium phosphate buffer (0.2 *M*, pH 5.1). The samples were chromatographed at room temperature at a flow-rate of 1 ml/min, 1000 p.s.i., a chart speed of 1 cm/min and monitored at 254 nm at 1.0 a.u.f.s.

RESULTS AND DISCUSSION

From Fig. 1 it is clear that good separation of each ribonucleotide and deoxyribonucleotide in each purine and pyrimidine series is achieved on a C_{18} column using isocratic elution with ammonium phosphate buffer (0.2 *M*, pH 5.1) at 1 ml/min flow-rate as previously described for the separation of ribonucleotides[1]. Following periodate treatment of the ribonucleotide–deoxyribonucleotide mixture, only deoxyribonucleotides and resulting free bases from ribonucleotides were detected.

In the chromatography of ribonucleotides, the order of elution is triphosphate followed by the diphosphate, with the monophosphate being retained for a relatively longer period. A similar pattern emerges with the deoxynucleotides. The order of

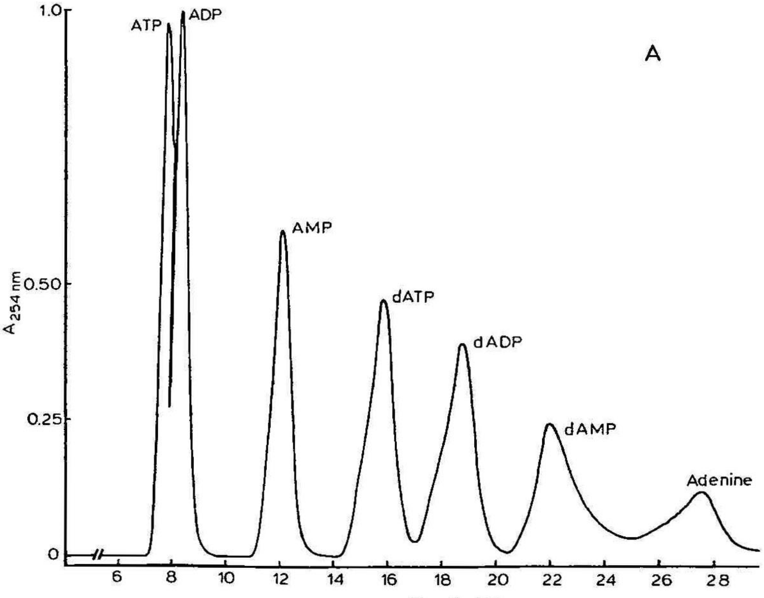


Fig. 1.

(*Continued on p. 310*)

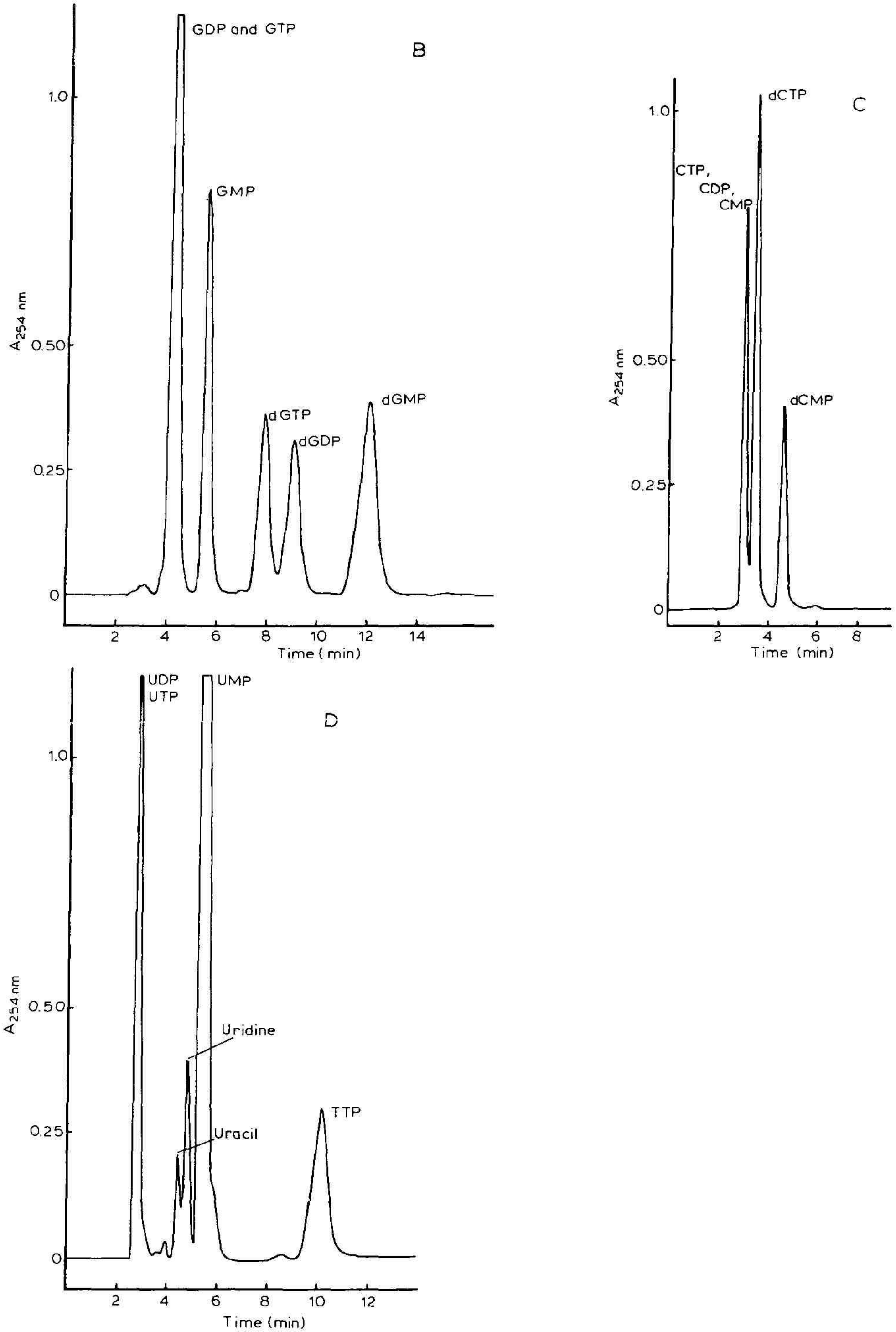


Fig. 1. Chromatogram of nucleotide standards on a μBondapak C_{18} column before periodate oxidation. A, Adenine series; B, guanine series; C, cytosine series; and D, uracil plus thymine series. Flow-rate, 1 ml/min.

elution of the ribonucleotides (uracil nucleotides before cytosine nucleotides before guanine nucleotides before adenine nucleotides) is also maintained for the deoxyribonucleotides. After periodate treatment, we have noted a minor shift in the elution pattern of the deoxyribonucleotides, the retention time of the adenine and guanine deoxyribonucleotides being slightly shorter than before the treatment.

Cyclic ribonucleotides under our conditions have a long retention time and are reasonably separated from the ribonucleotides and deoxyribonucleotides (cCMP at 5.9 min, cGMP at 36.4 min and cAMP at 112 min). However, the time of elution of cAMP is longer than normally desired with an HPLC system; modification of the eluent by the addition of differing amounts of methanol expedites the elution without affecting resolution (Fig. 2). The order of elution is consistent with that of the ribotides and deoxyribotides, *i.e.*, the cyclic ribotide of cytosine is retained least, that of guanine intermediate and adenine longest. From Fig. 2, it is also clear that the retention times of CTP, GTP and ATP are reduced by the incorporation of methanol in the eluent.

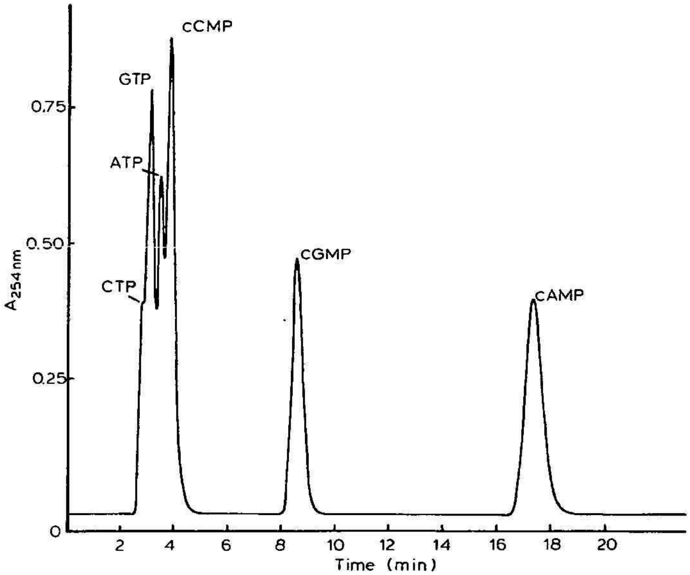


Fig. 2. Chromatogram of cyclic nucleotide standards on a μBondapak C_{18} column. The eluent was ammonium phosphate buffer (0.2 *M*, pH 5.1) containing 10% (v/v) methanol. Flow-rate, 1 ml/min.

The elution pattern of cytosine, uracil and thymine ribonucleotides and deoxyribonucleotides is similar, if not identical, making the resolution between tri- and diphosphates markedly difficult unless modifications are introduced in the eluting agent. This is in agreement with our previous results[1]. Yet from Fig. 1 it is obvious that each pyrimidine ribotide separates well from its corresponding deoxyribotide.

Five points emerge from this elution pattern: (i) the D-2-deoxyribose entity is retained on the column more tightly than the D-ribose entity, probably owing to the higher electronegativity of the OH group; (ii) the purine entity is retained on the column more tightly than the pyrimidine entity, probably owing to its higher charge;

(iii) the elution pattern in each series is clearly a function of the degree of protonation of the PO_4 group; the entity with the most PO_4 groups elutes earlier and the entity with the least elutes later; (iv) the degree of protonation is disproportionately masked (possibly for stereospecific reasons) by the number of phosphate groups, as is evident by the distance between the elution of tri- and diphosphates and monophosphates; and (v) cyclization of the monophosphates greatly increases their retention on the column. Thus the affinity of the nucleotides for the C_{18} column is not simply a function of the charge on the PO_4 group alone.

In order to test the applicability of this system to a biological sample, the total acid-soluble extract of HeLa cells was chromatographed as described under Experimental. Fig. 3 shows the nucleotide profiles. Although tentative identification of major peaks could be made by running standard nucleotides individually and by adding a standard to the mixture and confirming the peak by an increase in peak height, it was obvious that certain peaks contained more than one component. Measurement of differential retention times using phosphate buffer with and without 10% (v/v) methanol permitted their further identification. This approach proved exceedingly effective in the identification of cyclic nucleotides in HeLa cells (Fig. 4). Periodate oxidation allowed the clear identification of deoxyribonucleotides from ribonucleotides (Fig. 5). The periodate-treated sample also allowed the detection of bases whose retention times and peak areas remained unchanged as a result of periodate oxidation. The recovery of nucleotides was quantitative and the sensitivity of this method allowed us to measure samples at the level of nanomoles. Minor changes in retention time have been noted between columns, but the order of elution was consistent. A similar strategy of identification in conjunction with silica cartridge fractionation has been used successfully to identify and measure nucleotide concentration in *Stigmatella aurantiaca* and *Xenopus laevis* development[8].

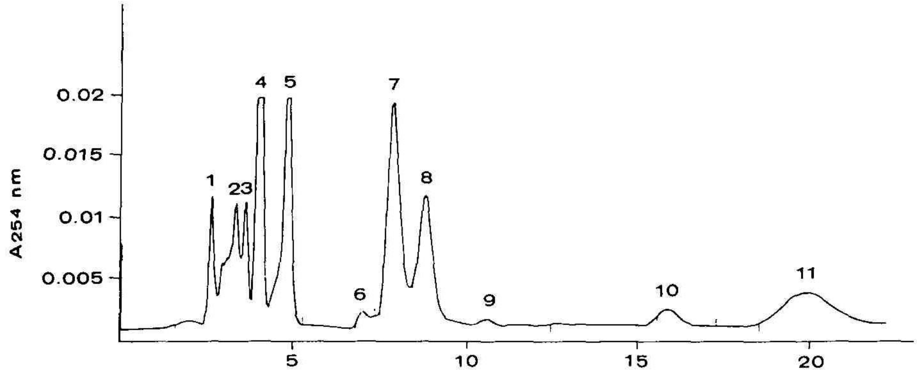


Fig. 3. Elution profile of total acid-soluble nucleotide pool from HeLa cells. All the peaks are identified individually: 1 = unidentified peak; 2 = UTP; 3 = CTP and CDP; 4 = GTP and UDP; 5 = GDP and UMP; 6 = GMP; 7 = ATP; 8 = ADP; 9 = hypoxanthine and guanine; 10 = AMP; 11 = dATP. Flow-rate, 1 ml/min.

Recent efforts to establish the applicability of metal chelate affinity chromatography to achieve the fractionation of AMP or GMP from their respective deoxy homologues were unsuccessful[9]. Besides the need to use a gradient, other procedures

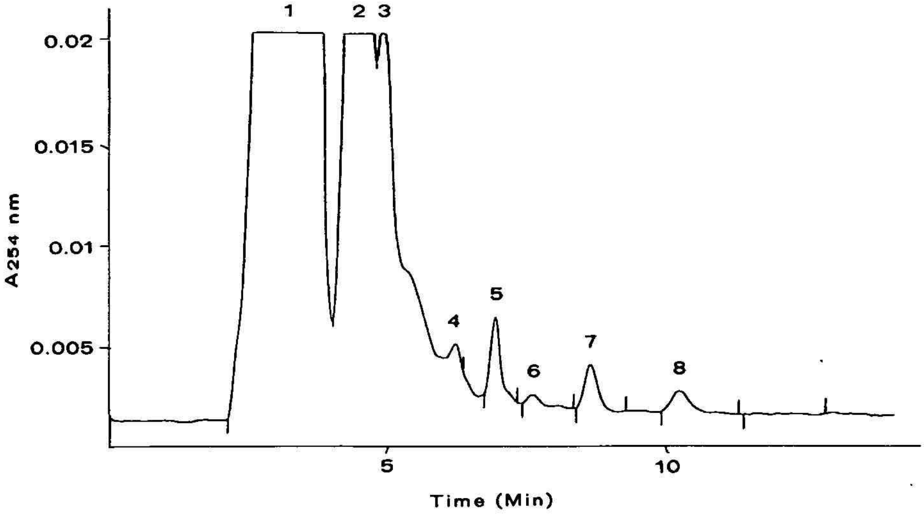


Fig. 4. Analysis of cyclic nucleotides in HeLa cells. Peak 1 contains at least four peaks as indicated by the integrator, composed of cytosine, uracil, cytidine and all nucleotides; peaks 2 and 3 contain hypoxanthine, guanine, and uridine; 4 = thymine; 5 = cGMP; 6 = guanosine; 7 = adenine; 8 = cAMP. Flow-rate, 1 ml/min.

showed coelution or overlapping elution of AMP, TMP and UMP[10]. The use of pH gradient[11], molarity gradient[12] or pH and molarity gradient techniques[13] required an increased time and buffer for equilibration between analyses and gradient programming for optimal resolution. None of these drawbacks are present in our procedure.

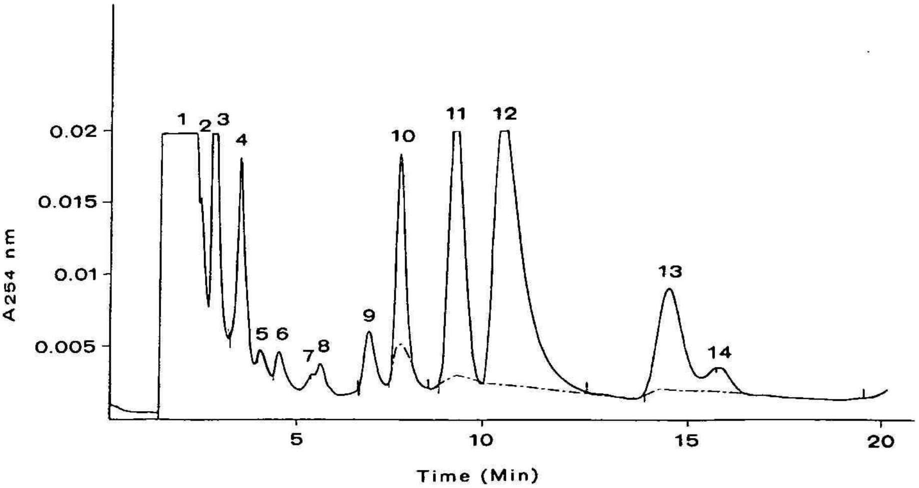


Fig. 5. Elution profile of periodate-treated nucleotide pool from HeLa cells. The cell extract was treated with periodate as described under Experimental. All peaks are identified individually: 1 = periodate; 2 = cyclohexylamine; 3 = uracil; 4 = unidentified; 5 = residual ATP; 6 = residual ADP; 7 = hypoxanthine; 8 = guanine; 9 = dGMP; 10 = dATP; 11 = dADP; 12 = adenine; 13 = dAMP; 14 = unidentified. Broken lines under peaks 10, 11, 13 and 14 represent the actual amounts and solid lines represent the coelution experiment. The increased adenine under peak 12 is due to periodate oxidation. Flow-rate, 2 ml/min.

It is convenient as it uses an isocratic mode of elution, thus avoiding the problems of gradient elution. It is simple, as it does not need the prior elaborate enzymatic treatments required in other systems[14]. Finally, it is comprehensive, as it affords clear and rapid separations of ribo-, deoxyribo- and cyclic nucleotide pools in cells under different pathological states. In the present instance, it has proved reliable and useful in the analysis of HeLa nucleotide pools.

ACKNOWLEDGEMENTS

This work was supported by grants GM18924 and AM 25498 from USPHS and in part by BRSG S07 RR7031 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health. The authors acknowledges the assistance of Mr. Garth E. Ziege in the preparation of the figures.

REFERENCES

1 M. W. Taylor, H. V. Hershey, R. A. Levine, K. Coy and S. Olivelle, *J. Chromatogr.*, 219 (1981) 133–139.
2 J. Maybaum, F. K. Klein and W. Sadee, *J. Chromatogr.*, 188 (1980) 149–158.
3 F. S. Anderson and R. C. Murphy, *J. Chromatogr.*, 121 (1976) 251–262.
4 M. McKeag and P. R. Brown, *J. Chromatogr.*, 152 (1978) 253–254.
5 A. Wakizaka, K. Kurosaka and E. Okuhara, *J. Chromatogr.*, 162 (1979) 319–326.
6 H. C. Neu and L. A. Heppel, *J. Biol. Chem.*, 239 (1964) 2927–2934.
7 C. D. Lothrop, Jr., and M. Uziel, *Anal. Biochem.*, 109 (1980) 160–166.
8 K. Clemens, R. M. Kothari, H. Martinez-Valdez, D. White and M. W. Taylor, in preparation.
9 P. Hubert and J. Porath, *J. Chromatogr.*, 206 (1981) 164–168.
10 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651–662.
11 R. T. Pon and K. K. Ogilvie, *J. Chromatogr.*, 205 (1981) 202–205.
12 P. D. Schweinsberg and T. L. Loo, *J. Chromatogr.*, 181 (1980) 103–107.
13 A. Floridi, C. A. Palmerini and C. Fini, *J. Chromatogr.*, 138 (1977) 203–212.
14 M. B. Cohen, J. Maybaum and W. Sadée, *J. Chromatogr.*, 198 (1980) 435–441.

Journal of Chromatography, 247 (1982) 315–325
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,054

SEPARATION OF PLANT HORMONES BY COUNTER-CURRENT CHROMATOGRAPHY*

N. B. MANDAVA*
U.S. Department of Agriculture, Agricultural Research Service, Plant Physiology Institute, Beltsville Agricultural Research Center, Beltsville, MD 20705 (U.S.A.)
and
Y. ITO
National Institutes of Health, National Heart, Lung and Blood Institute, Laboratory of Technical Development, Bethesda, MD 20205 (U.S.A.)

(First received April 29th, 1982; revised manuscript received May 24th, 1982)

SUMMARY

Counter-current chromatography (CCC) using the toroidal coil planet centrifuge was applied to the separation of plant hormones. Indole auxins were separated in either hexane–ethyl acetate–methanol–water (0.6:1.4:1.0:1.0) or chloroform–acetic acid–water (2:2:1). The latter solvent system was especially useful for the separation of abscisic acid from indole-3-acetic acid. Gibberellins (GA_3, GA_4 and GA_7) were separated from each other in ether–methanol–phosphate buffer (pH 7) (3:1:2). The CCC method was suitable for the separation of four cytokinins in ethyl acetate–methanol–phosphate buffer (pH 7) (3:1:3). Abscisic acid from Zoysia grass seed was successfully analysed by CCC. The potential practical application of CCC to plant hormone analysis is discussed.

INTRODUCTION

Plant hormones and other growth substances that are present in plant tissues at ppm and ppb** concentrations regulate the growth and development in all higher plants. They are required to study several metabolic changes, and other biochemical and physiological implications during the plant growth and developmental processes. Because of their presence in trace amounts, it is often very difficult to analyze and monitor their levels. Several analytical approaches including chromatographic spectroscopic methods have been suggested for their detection and analysis[1]. Recently, high-performance liquid chromatography (HPLC), gas chromatography with an

---

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

** In this article, the American billion (10^9) is meant.

electron-capture detector (GC–ECD) and selective ion monitoring (SIM) have been used to analyze various groups of plant hormones, especially to detect abscisic acid (ABA)* and indole-3-acetic acid (IAA), with reasonable success[2–4]. However, there are some inherent difficulties in these methods. Although HPLC has been found to be a superior method over other chromatographic systems, the choice of columns and solvent systems is limited, especially for separations of polar compounds and, moreover, other purification methods are needed prior to HPLC use for the determination or analysis of plant hormones from crude plant extracts. Both GC–ECD[5,6] and SIM methods[4–7] are very sensitive and can detect the trace substances at subnanogram levels. However, to utilize these methods, prior to introduction into the GC system, the compounds have to be converted into suitable derivatives in order to make them readily volatile and thus enable them to pass through the GC columns easily. Additionally, for SIM analysis, a thorough knowledge of mass spectrometry, suitable internal standards and above all a large investment of money in mass spectrometric (MS) instrumentation are required.

As an on-going project, we have been investigating analytical methods with a view to either refining the existing ones or developing new methods that would be better suited for plant hormone analysis. In this connection, we have examined a recently developed method, termed counter-current chromatography (CCC)[8], which is based on the principles of counter-current distribution (CCD) and liquid chromatography (LC). This paper deals with the application of CCC to the separation of standard mixtures of plant hormones and other related compounds.

MATERIALS AND METHODS

Instrument design

A simple tabletop model of the toroidal coil planet centrifuge[9,10] was used (Fig. 1). It has a rotary frame consisting of a pair of circular aluminum plates rigidly bridged with multiple aluminum links. The rotary frame is driven by a motor around the stationary pipe mounted on the central axis of the centrifuge and holds a pair of symmetrically spaced cylindrical holders (10 cm from the central axis), one of which (15 cm O.D.) has a coiled column while the other holder (10 cm O.D.) carries a counterweight to balance the centrifuge system. Each holder is equipped with a plastic gear (Winfred N. Berg, Inc.) which is coupled to an identical sun gear mounted around the central stationary pipe. This gear arrangement produces the desired planetary motion of the holders, *i.e.*, revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity in the same direction. For mechanical stability of the centrifuge, the free end (right side) of the rotary frame is coaxially connected to a short coupling pipe, which is supported by a stationary wall member of the centrifuge through a ball bearing.

The separation column is prepared by winding PTFE tubing (Zeus Industrial Products, Raritan, NJ, U.S.A.) of 0.55 mm I.D. on to a flexible core which is again

---

* Abbreviations: ABA = abscisic acid; GA_3 = gibberellic acid; GA_4 and GA_7 = gibberellins A_4 and A_7; IAA = indole-3-acetic acid; IA = indole-3-acetamide; IAN = indole-3-acetonitrile; IAcA = indole-3-acrylic acid; IBA = indole-3-butyric acid; ICA = indole-3-carboxylic acid; IPA_3 = indole-3-propionic acid; Z = zeatin; ZR =zeatin riboside; IP = 6-isopentenyladenine; IPA = 6-isopentenyladenosine.

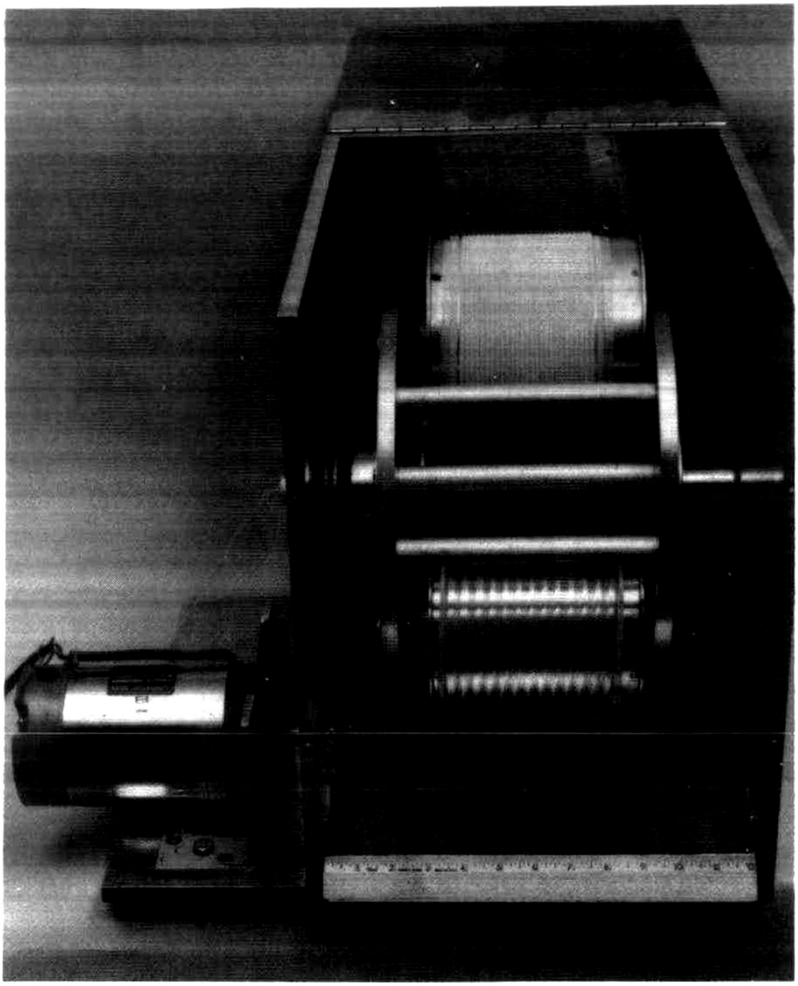


Fig. 1. A table-top model of the toroidal coil planet centrifuge.

coiled around the holder to make a coiled helix or toroidal coil configuration (helical diameter, 1.5 mm; total number of turns, 8500; total capacity, 18 ml; theoretical plates, 2000–6000). A counterweight is applied to the other holder to balance the centrifuge. The flow tubes from the column are first passed through the center of the coil holder shaft and then led into the coupling pipe through a side hole to enter the opening of the stationary pipe. To prevent mechanical damage, the flow tubes are lubricated (silicone grease) and protected with a piece of plastic tubing at each supported position. The planetary motion produced by the gears permits the flow tubes simply to roll around themselves without twisting. The revolutional speed is continuously adjustable up to 1000 rpm (450 *g*) with a Motomatic speed control unit (Electro-Craft).

Operation

The coiled column is first filled with a stationary phase of the pre-equilibrated two-phase solvent with the aid of either a Chromatronix Cheminert metering pump or a Milton-Roy pump. The test sample solution is introduced through a sample injection port and the mobile phase is pumped through the column at 2.4 ml/h while the apparatus is run at a desired revolutional speed (450–500 rpm). The eluate is continuously monitored with either an LKB Uvicord III or an LKB Uvicord S at 206, 260 or 280 nm, depending on the test samples, and then collected with a fraction collector.

Sources of hormone samples

All of the test compounds were obtained from several commercial sources and used without further purification. ABA-containing samples from Zoysia grass seeds were obtained by scarification of the seeds with alkali followed by extraction with diethyl ether–ethyl acetate (1:1). The crude acidic extract was purified by HPLC on a reversed-phase column Bondapak C_{18}, (Waters Assoc.) using methanol–water (80:20) and a fraction corresponding to the retention volume of standard ABA sample was collected and analyzed by CCC. Final proof for ABA was obtained by GC–MS of its methyl ester.

Partition coefficients

Partition coefficients were determined by adding a known amount of the test compounds in the solvent systems used for each group of hormones. After thoroughly mixing the compounds in the desired solvent system, the two phases were separated and the absorbances measured at the desired wavelength (indoles and ABA at 280 nm, cytokinins at 260 nm and gibberellins at 206 nm). The ratio of the absorbance values of the samples from two phases gave their partition coefficient (P.C.) in the respective solvent systems. A typical procedure for determining the P.C. of IAA in chloroform–acetic acid–water (2:2:1) is given here. To a mixture of 2 ml of chloroform, 2 ml of acetic acid and 1 ml of water were added 10 μl of IAA aqueous solution (concentration 20 μg/μl). The compounds and the solvents were mixed vigorously and two phases allowed to separate. A 1-ml volume of each phase was mixed with 3 ml of methanol and the absorbance was measured at 280 nm with a Beckman UV–visible spectrophotometer using a 1-cm light-path quartz cell which gave readings of 0.382 and 0.405 for the lighter phase (Lp) and the heavier phase (Hp), respectively. Hence the P.C. of IAA is 0.94 (Lp/Hp) for the above solvent system.

RESULTS AND DISCUSSION

Indole compounds

Two chromatographic solvent systems, I (hexane–ethyl acetate–methanol–water, 0.6:1.4:1:1) and II (chloroform–acetic acid–water, 2:2:1), were chosen for separation of several indolic compounds by CCC. The partition coefficients (Table I) range from 0.26 to 3.83 in these two solvent systems. The compounds studied either exhibit auxin activity or are the metabolic products of IAA. Because of the inherent differences in their P.C.s in these solvent systems, one would expect these indoles from a mixture to separate into individual components by CCC. If the upper phase is kept

TABLE I

PARTITION COEFFICIENTS OF INDOLES IN TWO DIFFERENT SOLVENT SYSTEMS

Compound	*Abbreviation*	*Concentration (μg/μl)*	*Partition coefficient*			
			Solvent system I		*Solvent system II*	
			Lp/Hp	*Hp/Lp*	*Lp/Hp*	*Hp/Lp*
Indole-3-acetic acid	IAA	20	0.99	1.01	0.94	1.06
Indole-3-acetamide	IA	20	0.26	3.83	1.05	0.95
Indole-3-acetonitrile	IAN	20	3.00	0.33	0.39	2.55
Indole-3-carboxylic acid	ICA	10	0.63	1.61	1.07	0.93
Indole-3-acrylic acid	IAcA	10	0.88	1.44	0.78	1.28
Indole-3-butyric acid	IBA	20	1.74	0.57	0.36	2.81
Indole-3-propionic acid	IPA_3	20	2.02	0.50	0.26	3.80

Solvent system I, hexane–ethyl acetate–methanol–water (0.6:1.4:1:1); solvent system II, chloroform–acetic acid–water (2:2:1). Hp, heavier phase; Lp, lighter phase.

stationary on the column and the lower phase is used to elute the column, the partition coefficients (Hp/Lp) for these compounds (Table I, columns 5 and 7) serve as a useful guide for predicting the order of elution for these compounds in a mixture. When the phases are reversed, one must then use columns 4 and 6 of Table I. As the elution pattern follows the decreasing order of P.C.s, the separation of the mixture begins with the highest P.C.-containing compound followed by compounds with lower P.C.s.

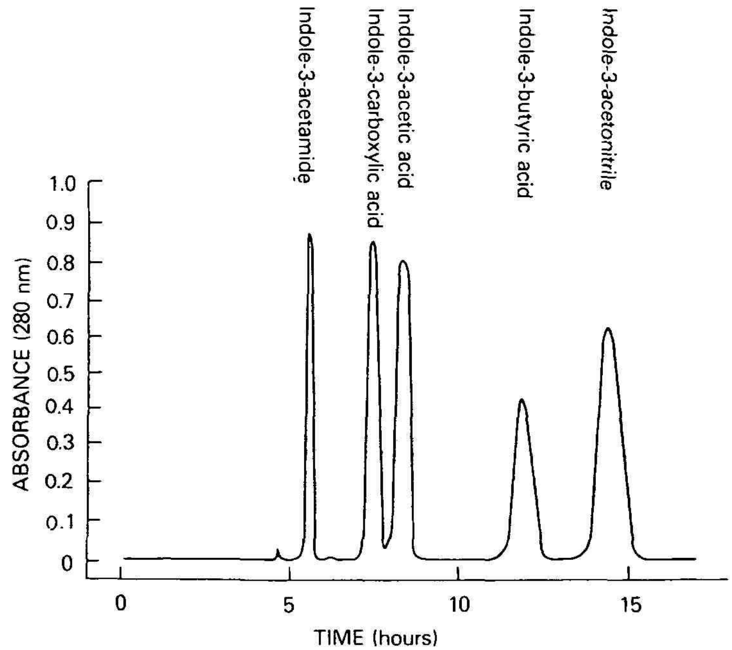


Fig. 2. Separation of indole compounds by CCC in hexane–ethyl acetate–methanol–water (0.6:1.4:1:1); mobile phase: aqueous phase.

A mixture (25 μg) containing five indoles (IAA, IA, IAN, ICA and IBA; 50 μg each) whose P.C.s vary from 0.33 to 3.83 were chromatographed in solvent system I, keeping the lighter (non-aqueous) phase stationary. These compounds eluted in the order that one would predict: IA (P.C. 3.83) eluted first followed by ICA (1.61), IAA (1.01), IBA (0.57) and IAN (0.33) with the lower phase as mobile phase (Fig. 2). In solvent system II, a mixture containing four indoles (ICA, IAcA, IBA and IPA$_3$) and ABA was analyzed by CCC. IAA was not introduced in this mixture because it would obscure the resolution of ICA and IAcA peaks as it falls between the two peaks (resulting in poor resolution of peaks). Keeping the organic (heavier) phase stationary, all five compounds were separated with the lighter (aqueous) phase solvent (Fig. 3). A small peak just before the ABA peak was observed and was identified as an impurity of the IPA$_3$ sample. ABA, which has a P.C. (Lp/Hp) of 0.56, eluted after IAcA (0.78). IAA and ABA were easily separated using the same conditions (Fig. 4) because of the differences in their P.C.s in the solvent system II. These results clearly indicate that the indole compounds that are commonly found in plant tissue can be separated with solvent system I and/or II. Although IBA (0.57) and IPA$_3$ (0.50) could not be separated in solvent system I because of their close P.C.s, they were clearly resolved using solvent system II. ABA present in plant samples could also be detected and separated from IAA by CCC with solvent system II, which is especially useful for the separation of ABA from indoles (because of the difference in their P.C.s). These results suggest that, by a judicious choice of a solvent system, indole compounds as well as other plant metabolites can be readily separated in the presence of ABA.

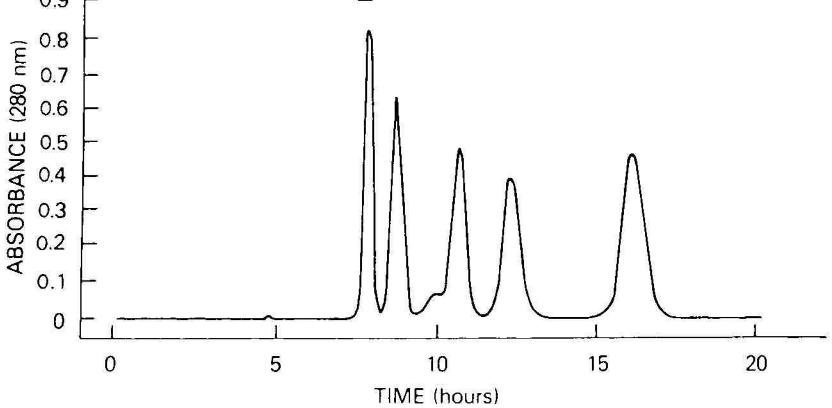


Fig. 3. CCC separation of a mixture of four indolic carboxylic acids and abscisic acid in chloroform–acetic acid–water (2:2:1), retaining organic phase stationary.

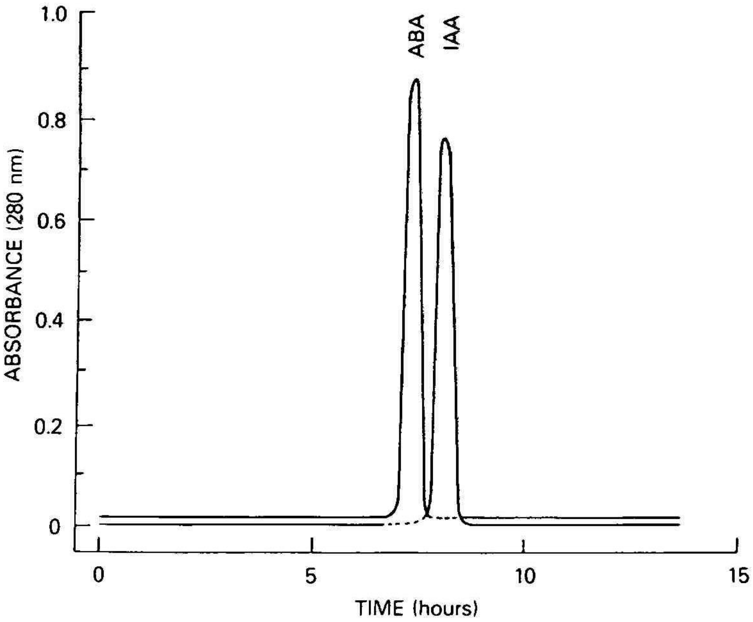


Fig. 4. CCC separation of indole-3-acetic acid and abscisic acid in chloroform–acetic acid–water (2:2:1), retaining organic phase stationary.

Gibberellins

As in HPLC, the separation of this group of plant hormones presented problems, mainly because of the lack of a proper detection system. None of the gibberellins separated here showed UV absorption between 220 and 360 nm. However, all of them had strong end absorption around 210 nm, which was used to monitor them with a UV detector. The choice of solvents was limited because gibberellins do not partition very well in the ordinarily employed solvent systems. A solvent system containing ethyl acetate–phosphate buffer (pH 7) was reported previously to be an ideal system in the countercurrent distribution of gibberellin[11]. As ethyl acetate (UV cut-off at 230 nm) interferes with UV monitoring at 210 nm, diethyl ether was used in its place. The use of the diethyl ether–0.50 *M* phosphate buffer system had the disadvantage that it has a very high interfacial tension, which creates a high column pressure.

This difficulty was eliminated by introducing methanol into the system. A solvent system containing diethyl ether–methanol–0.5 *M* phosphate buffer (pH 5.9) (3:1:2) was suitable for gibberellin separation. A GA_3 and GA_4–GA_7 mixture was used for separation work. With the lighter (organic) phase mobile, GA separation was achieved (Fig. 5). Under these conditions, GA_3 eluted first, followed by GA_7 and finally GA_4. The total run took less than 8 h, although we allowed the chromatograph to run for 24 h. To confirm that the chromatographic peaks contain gibberellins, all the fractions were bioassayed in a bean second internode test[12], which showed activity for those fractions that represented the peaks. Thus, the correlation of the peaks with activity was made for the separated gibberellins.

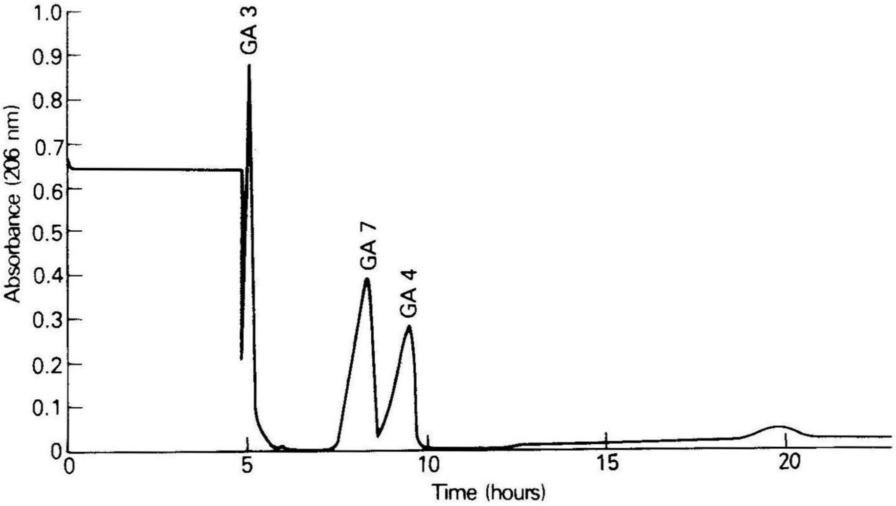


Fig. 5. Separation of gibberellins A_3, A_4/A_7 by CCC; solvent system: ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 5.9 (3:1:2); mobile phase: organic phase.

Cytokinins

This method is especially useful for separation of cytokinins, namely for separating a mixture of Z, ZR, IP and IPA, as they were well resolved in a solvent system containing ethyl acetate, methanol and phosphate buffer. A change of pH from 5 to 9 affected their partition coefficients considerably when they were partitioned between ethyl acetate and 0.5 *M* phosphate buffer[13]. For example, ZR showed a P.C. (C_{aq}/C_{org}) range of 8–10 whereas IPA has P.C.s of 0.08–0.1 in this solvent system. The solvent combination of our choice was ethyl acetate–methanol–0.5 *M* phosphate buffer (pH 7) (3:1:3) in which the desired range of P.C.s (0.2–5.0) for these compounds was accomplished (Table II). The cytokinin mixture, when subjected to CCC separation keeping the organic phase stationary, was completely resolved, according to the decreasing order of their P.C.s. (C_{aq}/C_{org}); *i.e.*, ZR which has the highest P.C., eluted first, followed by Z, IPA and IP (Fig. 6). However, the Z and ZR peaks appeared too close, whereas IP and its riboside (IPA) were separated farther apart (because of the long retention times). The commercial samples of Z and ZR contained

TABLE II

PARTITION COEFFICIENTS OF CYTOKININS IN ETHYL ACETATE–METHANOL–0.5 *M* PHOSPHATE BUFFER (pH 7) (3:1:3)

Cytokinin	*Abbreviation*	*Partition coefficient*	
		C_{aq}/C_{org}	C_{org}/C_{aq}
Zeatin riboside	ZR	4.25	0.24
Zeatin	Z	2.26	0.44
6-Isopentenyladenosine	IPA	0.73	1.37
6-Isopentenyladenine	IP	0.20	5.00

impurities which were found in the chromatogram before, after and between these peaks. In order to make the Z and ZR peaks well resolved one should adapt by either simply reversing the phase in the CCC run or choosing an entirely different solvent system, so that the P.C.s for these compounds appear below 1.0 and preferably around 0.5. We have chosen to use the same solvent system but simply to reverse the phase, whereby the change in P.C.s (C_{aq}/C_{org}) reverses the order of the elution pattern for these compounds (Fig. 7). In this way, IP, which had the longest retention time in the above situation (see Fig. 6), is now eluted with the shortest retention time whereas ZR had the longest retention time (Fig. 7). Further, Z and ZR were well separated (ΔR_t = 5 h) and thereby the impurities in the mixture did not interfere with the Z and ZR peaks.

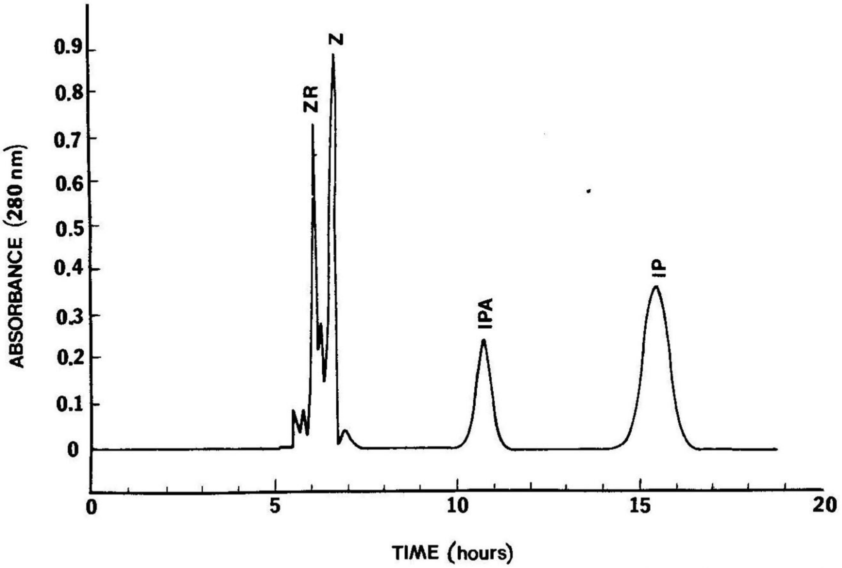


Fig. 6. Separation of zeatin and 6-isopentenyl adenine and their ribosides in ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 7 (3:1:3); stationary phase: non-aqueous phase.

This approach demonstrates that (1) the partition coefficients either directly or inversely relate to the retention times measured from the solvent front and (2) reversal of the choice for the mobile phase gives the reciprocal values of the P.C.s and therefore affects the retention times of the solute molecules. The approach has the following advantages. (1) Changing the phase from stationary to mobile is very easy to perform in CCC. (2) If particular compound takes several hours to elute, changing the phase reduces the retention time considerably. (3) In plant sample mixtures (containing cytokinins), one could first separate the hormones from other undesired compounds by a process where they can be collected first (with shorter or long retention times) and later, by reversal of the phase in CCC, one could obtain a pure compound.

Abscisic acid analysis

In connection with our work on seed germination, we have investigated Zoysia

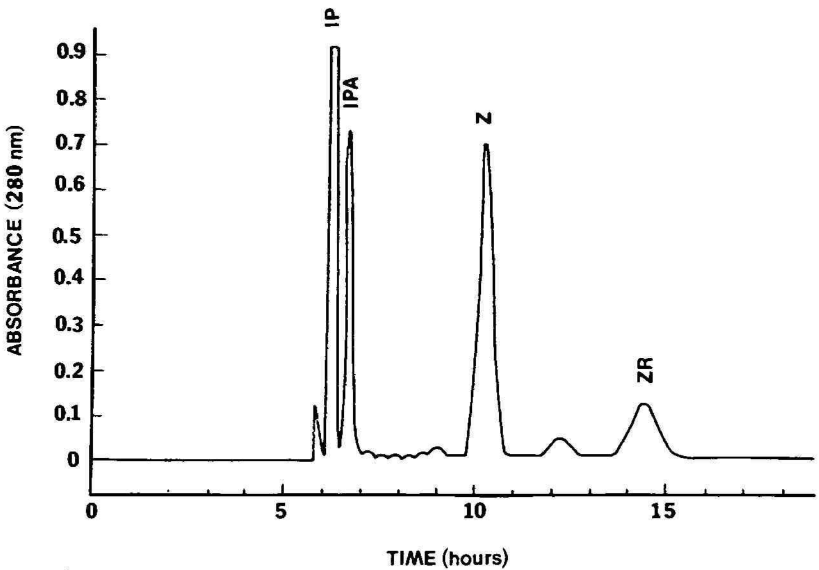


Fig. 7. Separation of cytokinins in ethyl acetate–methanol–0.5 *M* phosphate buffer, pH 7 (3:1:3); stationary phase: aqueous phase.

grass seed, which has a tendency to remain dormant but scarification with alkali for a few minutes[14] allowed the seed to germinate easily. We extracted the scarified seed with diethyl ether–ethyl acetate and chromatographed the extract by HPLC. A fraction corresponding to the retention of standard ABA was collected and subjected to CCC separation. This fraction showed a single peak that corresponded to the retention time of the standard ABA. Thus the confirmation for ABA in the plant extract was obtained by comparing it in two chromatographic systems followed by MS analysis of the corresponding methyl ester.

Advantages of CCC over CCD and LC

In comparison with CCD, CCC offers the following advantages: (1) much higher partition efficiency; (2) shorter separation times; (3) separation of small amounts of samples without much dilution; (4) little or no risk of emulsification; (5) a wide selection of solvents which are required in relatively small amounts; (6) separation of compounds whose P.C.s fall between 0.2 and 5; (7) molecules of any size can be separated; (8) the apparatus is much smaller and less expensive (*ca.* $5000); (9) samples are not exposed to the air and hence there is no deterioration of sensitive compounds; and (10) continuous monitoring of the eluate is possible with a conventional UV detector. The main disadvantage of CCC compared with CCD is that the sample sizes are limited to a few hundred milligrams, unless larger bore coils are employed in CCC.

A few obvious advantages over LC are (1) sample loss is minimal and even the samples precipitated in the column can be recovered easily; (2) sample deterioration is minimal; (3) there is no tailing of the solute peaks due to the adsorption effects of the solid support; (4) there is no contamination due to the use of solid supports; (5) high reproducibility; (6) prediction of the location of the solute peaks is possible simply by measuring the P.C.s of the sample; (7) the system provides the choice of the mobile phase, either aqueous or non-aqueous and either upper or lower phase; (8) the large amount of the stationary phase allows a large sample loading capacity; and (9) the column is easily cleaned and can be reused almost indefinitely. One major disadvantage of CCC over LC is the separation time, which is very long; it takes at least 10 h to yield several thousand theoretical plates, whereas HPLC can produce comparable separations in less than 1 h. Despite the limitation of long separation times, CCC appears to be useful for the separation of polar compounds from the crude mixtures. It will not replace conventional chromatographic methods such as TLC, GC and HPLC, but is complementary. When used alone or in conjunction with these methods, CCC is of great help for the separation of plant hormones.

CONCLUSIONS

The limited work presented in this paper demonstrates that CCC appears to have great potential for the separation of plant hormones and other growth substances in an efficient manner. Neither expensive columns nor costly instruments are required in this type of separation. With the availability of different CCC prototypes, separation can be carried on on either an analytical or a preparative scale. When desired, gradient elution can be performed in a manner similar to HPLC. In retrospect, this method may have wide applicability for the total hormonal analysis of plant samples after conventional extraction methods give acidic, basic and neutral fractions, each of which could be successfully analyzed following the procedures outlined above.

REFERENCES

1 M. L. Brenner, in N. B. Mandava (Editor), *Plant Growth Substances*, Vol. 111, ACS Symposium Series, American Chemical Society, Washington, DC, 1979, Ch. 8, p. 215.
2 J. R. Hillman (Editor), *Isolation of Plant Growth Substances (Society for Experimental Biology Seminar Series 4)*, Cambridge University Press, Cambridge, 1978, p. 157.
3 N. Mandava, *Amer. Lab.*, 5 (1973) 27.
4 N. Mandava, *Proc. Ninth Materials Research Symposium on Trace Organic Analysis (NBS Special Publication No. 519)*, National Bureau of Standards, Washington, DC, 1979, p. 803.
5 A. Ciha, M. L. Brenner and W. A. Brun, *Plant Physiol.*, 59 (1977) 821.
6 F. Whitman, in T. K. Scott (Editor), *Plant Growth Regulation and World Agriculture*, Plenum Press, New York, 1979, p. 327.
7 C. Rivier, H. Milan and P. E. Pilet, *Planta*, 134 (1977) 23.
8 Y. Ito, *J. Biochem. Biophys. Methods*, 5 (1981) 105.
9 Y. Ito, *Anal. Biochem.*, 102 (1980) 150.
10 Y. Ito, *J. Chromatogr.*, 192 (1980) 75.
11 R. C. Durley and R. P. Pharis, *Phytochemistry*, 11 (1972) 317.
12 J. W. Mitchell and G. A. Livingston, *Agricultural Handbook, No. 336*, Agricultural Research Service, U.S. Department of Agriculture, U.S. Government Printing Office, Washington, DC, 1968.
13 T. Hemberg, *Physiol. Plant*, 32 (1974) 191.
14 D. Y. Yeam, unpublished results.

Journal of Chromatography, 247 (1982) 327–334
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,060

THIN-LAYER CHROMATOGRAPHY WITH FLAME-IONIZATION DETECTION OF CHLOROMETHANESULPHONANILIDE AND ITS ETHOXYCARBONYLMETHYL DERIVATIVES

M. RANNÝ*, M. ZBIROVSKÝ, M. BLÁHOVÁ and V. RŮŽIČKA
Department of Organic Technology, Prague Institute of Chemical Technology, Prague (Czechoslovakia)
and
Š. TRUCHLIK
Research Institute of Agrochemical Technology, Bratislava (Czechoslovakia)

(Received May 25th, 1982)

SUMMARY

A thin-layer chromatographic method with flame-ionization detection for the quantitative evaluation of mixtures from a reaction of chloromethanesulphonanilide with ethyl chloroacetate is described. The best results were obtained using Chromarods S II with *n*-hexane–dichloromethane–methanol (25:25:0.25 as the solvent system). The good accuracy and reproducibility in the range 2.5–100 μg suggest that this method could be of appreciable value for the analysis of compounds with chlorine and sulphur in the molecule, *e.g.*, for monitoring syntheses of pesticides.

INTRODUCTION

The combined use of thin-layer chromatography with a flame-ionization detection (TLC–FID) represents a significant advance as a method applicable to screening in biomedical and pharmaceutical areas[1,2]. This sensitive, relatively simple and rapid procedure also offers advantages in characterizing surfactants[3], fuel oils[4] and food additives[5], as well as for industrial research[6] analyses.

This paper reports our experiences with the TLC–FID technique in analysing products from the reaction of chloromethanesulphonanilide with ethyl chloroacetate. (Ethoxycarbonylmethyl)chloromethanesulphonanilide, appears to have a considerable application potential in herbicide preparations[7].

EXPERIMENTAL

Reagents and chemicals

Chloromethanesulphonanilide (CMSA) and N-(ethoxycarbonylmethyl)chloromethanesulphonanilide (Et-CMSA), prepared in our Institute, were at least 99% pure, melting points 74 and 51°C, respectively. Ethyl stearate (ES) (puriss.) was obtained from Fluka (Buchs, Switzerland).

All solvents were of reagent grade and were dried and distilled in glass before use.

0021-9673/82/0000–0000/$02.75

Apparatus and conditions

The Iatroscan TH-10 Analyser, Mark II (Iatron Labs., Tokyo, Japan; distributed by Newman-Howells Assoc., Winchester, Hants., Great Britain) was connected to a Spectra-Physics Computing Integrator System I and a Linseis LS 24700 single-pen linear recorder.

Glass developing tanks (Iatron) were lined with Whatman No. 2 filter-paper which was pre-washed with mobile phase A or B (see below).

The stationary phase was Chromarod S II (Iatron) and the mobile phase were (A) *n*-hexane–benzene–methanol (40:10:0.5) and (B) *n*-hexane–dichloromethane–methanol (25:25:0.25). The scanning speed was 0.31 cm/sec (30-tooth gear), the chart speed 100 cm/min and the recorder range 100–800 mV full-scale.

Standard test mixtures

Seven standard solution mixtures were prepared for evaluation purposes. They included ethyl stearate as an internal standard and CMSA and Et-CMSA in various proportions (Table I).

TABLE I

COMPOSITION OF STANDARD SOLUTIONS

The concentration of ES was 5.0 μg/μl in all mixtures.

Mixture No.	*Concentration (μg/μl)*		*Weight-%*		
	Et-CMSA	*CMSA*	*ES*	*Et-CMSA*	*CMSA*
1	10.0	0.5	32.3	64.5	3.2
2	5.0	2.5	40.0	40.0	20.0
3	2.5	5.0	40.0	20.0	40.0
4	1.0	10.0	31.3	6.2	62.5
5	2.1	2.4	52.6	22.1	25.3
6	30.0	29.3	7.8	46.6	45.6
7	0.5	10.0	32.3	3.2	64.5

Procedure

A new set of Chromarods was twice blank-scanned in the FID burner and then immersed for 12 h in concentrated sulphuric acid. Prior to use, the rods were washed five times with *ca.* 200 ml of distilled water, dried for 10 min in a glass oven at 130°C and then subjected to a final single blank-scan. The activated Chromarods were then spotted with 1-μl portions of sample or standard solutions and developed for 30 min in one of the above-mentioned solvent mixtures. After oven-drying for 7 min at 100°C, the Chromarods were transferred to a scanning frame for detection.

Treated Chromarods whose low "noise" uptake was apparent or the separating capacity of which appeared to deteriorate (after *ca.* 5–7 analyses) were cleaned by immersion for 1 h in 6 *N* hydrochloric acid and by 5–6 subsequent washings with distilled water.

RESULTS AND DISCUSSION

Separation and R_F values

Of the many developing solvents previously tested, two appeared to be most suitable. As shown in Fig. 1a and b and in Table II, solvent B seems to be preferable because of a better baseline (lower FID noise) and good resolution of the three components in the central zone of the chromatogram, all of which provided a realistic basis for better reproducibility of analyses.

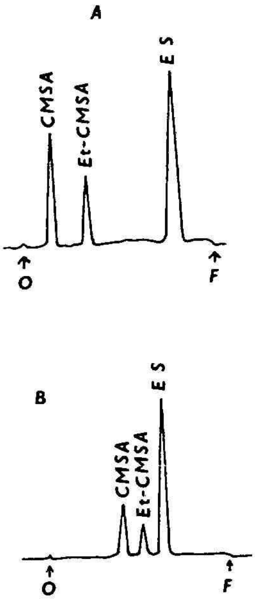


Fig. 1. Chromatogram of standard mixture No. 3. (A) Solvent system A; (B) solvent system B. Recorder range: 200 mV full-scale.

TABLE II

R_F VALUES OF Et-CMSA, CMSA AND THE INTERNAL STANDARD (ES) IN SOLVENT SYSTEMS A AND B

Solvent	*ES*	*Et-CMSA*	*CMSA*
A	0.81	0.35	0.16
B	0.65	0.52	0.42

Response of the FID and evaluation of the analyses

It is known in gas–liquid chromatography with an FID that the response of organic compounds depends on the proportion of carbon atoms that contain little or no oxygen[8]. This relationship need not be valid in TLC–FID, where the absolute weight response is a complex question involving both mechanical and chemical effects[1], *e.g.*, the operating parameters of the detector[1,9], the relative volatility of the separated organic matter, the interaction between the organic material and adsorbent[10] and the shape and the position of the separated components on the Chromarod[8].

Nevertheless, under strictly controlled conditions, it may be assumed that FID responses are reproducibly proportional to the amounts of the components spotted. This relationship is presented in eqns. 1–3 from the analyses of standard mixtures (Table I) on seven different rods by means of the least-squares method using a digital computer:

$$A_{IS} = 18x_{IS} \ (r = 0.998) \tag{1}$$

$$A_{EA} = -9.6 + 12.1x_{EA} \ (r = 0.990) \tag{2}$$

$$A_{A} = -10.1 + 8.8x_{A} \ (r = 0.998) \tag{3}$$

where A_i is the relative area of a particular peak (the reading from the computing integrator × 10^{-3}), x_i is the amount spotted, the subscripts EA and A correspond to Et-CMSA or CMSA, respectively, and r is the regression coefficient.

As can be seen, the characters of the calibration straight lines of the internal standard and of the two biocides are slightly different; the former line passes through the origin, whereas the latter two intersect the amount axis at 0.8 μg (Et-CMSA) and 1.1 μg (CMSA).

From the regression equations it can be concluded that $K_{FID(lim)}$ are about 1.5 for Et-CMSA and 2.1 for CMSA (K_{FID} of the internal standard = 1.0) (Fig. 2).

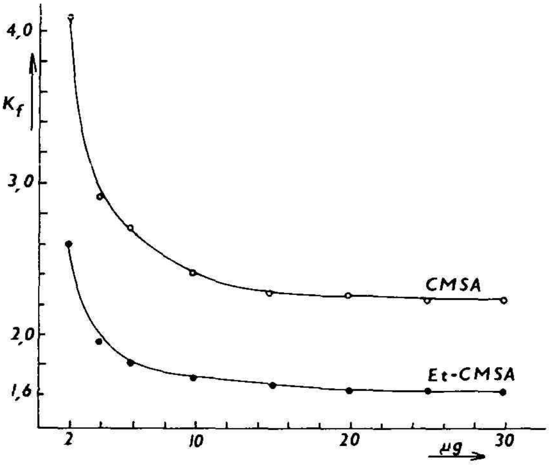


Fig. 2. Plot of K_{FID} *versus* amount spotted.

From a comparison with theoretical K_{FID} values, calculated from the molar percentages of carbon in the analysed components, one can deduce that the response of CMSA seems to be proportional to the content of aromatic carbons or, in other words, the carbon atoms that are bound to chlorine or sulphur do not contribute to the total response. However, the response of Et-CMSA is higher than that which corresponds to the content of total carbon atoms in the molecule (Table III).

TABLE III

THEORETICAL K_{FID} VALUES

K_{FID} = reciprocal response

Type of carbon	*Et-CMSA**		*CMSA**	
	A	*B*	*A*	*B*
All C	1.67	1.56	1.73	1.65
Aromatic C	1.95	1.71	2.02	1.92

* A, calculated from the percentage of the total carbon atoms in the internal standard (76.9%); B, calculated from the percentage of non-carbonyl carbon atoms in the internal standard (73.1%).

At this stage, no attempt has been made to provide an explanation for the different behaviour of the two compounds, which illustrates the complexity of FID responses in the TLC–FID technique.

From eqns. 1–3, the relationships for the concentration c_i (in mg/ml) or percentage P_i of a component i in the sample solution can be written as follows:

$$c_{EA} = \frac{x_{EA} c_{IS}}{x_{IS}} \tag{4}$$

$$c_{A} = \frac{x_{A} c_{IS}}{x_{IS}} \tag{5}$$

$$P_{EA} = \frac{x_{EA}}{x_{EA} + x_{A}} \cdot 100 \tag{6}$$

$$P_{A} = \frac{x_{A}}{x_{EA} + x_{A}} \cdot 100 \tag{7}$$

The values of c_{IS} and x_{IS} are known.

Accuracy and reproducibility

The accuracy of the method was checked by comparing theoretical values with averaged data, obtained from seven replicate analyses of standard mixtures 1–7 (see Table I). The results are summarized in Table IV.

Reproducibility was determined from the same experimental data by calculating the coefficients of variation (C.V.) (Table V).

It was shown that, within the range 2.5–30 μg, the experimental values are sufficiently accurate and differ from the theoretical values by only *ca.* 3%. At lower concentrations, however, the relative accuracy was strongly reduced and the corresponding deviations were, in some instances, greater than 100%, *i.e.*, the validity of the equations for Et-CMSA and CMSA is limited to values of x_i greater than 2.5 μg.

TABLE IV

COMPARISON OF THE THEORETICAL AND EXPERIMENTAL COMPOSITIONS OF STANDARD MIXTURES 1–7 (TABLE I)

Mixture No.	*ES (wt.-%)*		*Et-CMSA (wt.-%)*		*CMSA (wt.-%)*	
	Theoretical	*Experimental*	*Theoretical*	*Experimental*	*Theoretical*	*Experimental*
1	32.3	31.0	64.5	61.1	3.2	7.9
2	40.0	41.2	40.0	39.4	20.0	19.4
3	40.0	41.1	20.0	19.3	40.0	39.6
4	31.3	29.8	6.2	8.9	62.5	61.3
5	52.6	51.3	22.1	21.2	25.3	27.5
6	7.8	7.5	46.6	47.1	45.6	45.4
7	32.3	31.2	3.2	7.1	64.5	61.7

TABLE V

COEFFICIENTS OF VARIATON *VERSUS* THE AMOUNT OF Et-CMSA AND CMSA SPOTTED

Amount of ES spotted = 5 μg.

Mixture No.	*ES C.V. (%)*	*Et-CMSA*		*CMSA*	
		μg	*C.V. (%)*	*μg*	*C.V. (%)*
1	2.2	10.0	1.6	0.5	48.0
2	2.8	5.0	0.9	2.5	10.3
3	1.9	2.5	3.8	5.0	3.5
4	4.1	1.0	6.8	10.0	1.8
5	2.6	2.1	4.1	2.4	4.8
6	2.9	30.0	1.4	29.3	1.5
7	3.2	0.5	14.4	10.0	1.3

Similarly, the reproducibility of the calibration data also depends on the amount of substance analysed. In the concentration range below 2 μg it was poor (C.V. 10–50 %); above 10 μg, however it was very good (C.V. *ca.* 1–2 %), *i.e.*, comparable to, or even better than, that of acylglycerols and fatty acids[11].

The lower accuracy and reproducibility in the range 0.5–2 μg could be deduced from the non-linear dependence of the FID response on the loading of the rod. This behaviour could be caused not only by incorrect setting of the parameters of the computing integrator (*i.e.*, the preference of the small peaks which could be integrated together with part of the baseline), but also by the influence of elements other than carbon and hydrogen in the analysed material[1] and those in the thin layer.

The amount of sample accepted by the rod without any deterioration of separation and signal deformity may be influenced by many variables, including the separation ability of the Chromarod in a particular solvent system, the number of components in an analysed mixture and the chemical constitution of the separated compounds.

It was found advantageous to keep the amount of a single component spotted in the range 1–10 μg. Nevertheless, in mixtures containing minor components it might be necessary to operate with higher loads in order to obtain sufficient accuracy and reproducibility at small concentrations. Included in such instances are reaction mixtures of Et-CMSA and CMSA, especially those taken from the starting or final period of the synthesis.

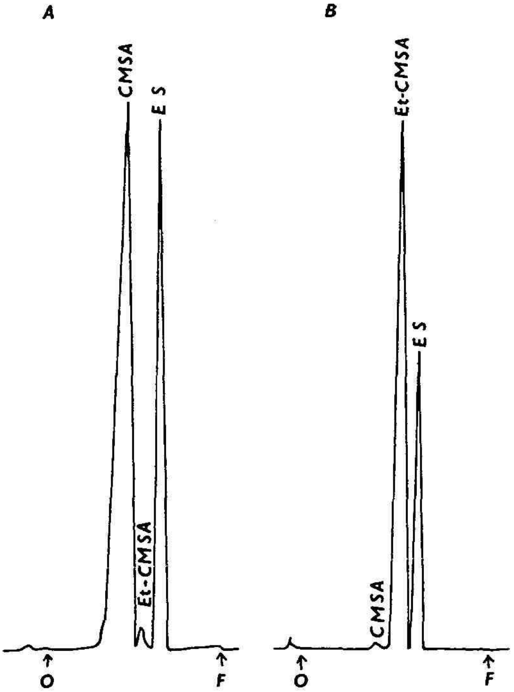


Fig. 3. TLC–FID analysis of the reaction mixtures. (A) Starting period of the reaction; (B) final period of the reaction. Solvent system B. Recorder range: 800 mV full-scale.

TABLE VI

TLC–FID ANALYSES OF SAMPLES FROM THE REACTION OF CMSA WITH ETHYL CHLOROACETATE

Analysis No.	*Starting period (wt.-%)*			*Final period (wt.-%)*		
	ES	*Et-CMSA*	*CMSA*	*ES*	*Et-CMSA*	*CMSA*
1	17.07	1.66	81.27	19.08	78.93	1.99
2	17.48	1.57	80.95	19.78	78.38	1.84
3	16.56	1.32	82.12	19.76	78.20	2.04
4	17.54	1.53	80.93	19.18	78.80	2.02
5	17.74	1.39	80.87	20.34	77.55	2.11
6	17.30	1.47	81.23	20.12	77.90	1.98
7	17.42	1.63	80.95	19.37	78.41	2.22
Mean	17.30	1.51	81.18	19.66	78.31	2.03
C.V. (%)	2.5	8.3	0.6	2.4	0.6	6.9

The results of the analyses of two such mixtures are shown in Fig. 3 and Table VI.

In these analyses, each rod was spotted with *ca.* 120 μg of the dry substance, *i.e.*, with about 100 μg of the major and 2 μg of the minor component. In spite of such high loads, the separation of all the peaks was sufficient and the coefficient of variation of the minor component was well below 10 % and that of the major components even below 1 %.

TABLE VII

COMPARISON OF TRUE AND OBSERVED COMPOSITIONS OF STANDARD SOLUTIONS AT HIGHER LOADINGS

Average values of seven replicate determinations.

Mixture No.	*Compound*	*Amount spotted (μg)*	*True composition (wt.-%)*	*Observed composition (wt.-%)*	*C.V. (%)*	*Absolute error (wt.-%)*	*Relative error (%)*
8	ES	20.6	16.7	17.2	3.7	+0.5	2.9
	Et-CMSA	1.6	1.3	1.1	22.0	−0.2	15.4
	CMSA	101.5	82.0	81.7	0.6	−0.3	0.4
9	ES	19.9	16.5	16.9	1.8	+0.4	2.4
	Et-CMSA	98.0	81.0	80.8	0.5	−0.2	0.2
	CMSA	3.0	2.5	2.3	8.0	−0.2	8.0

To ascertain whether eqns. 1–3 are valid also at these high spotted amounts, two further standard solutions were prepared. The results given in Table VII are sufficiently precise and accurate to show the applicability of these relationships.

REFERENCES

1 R. G. Ackman, *Methods Enzymol.*, 72 (1981) 205.
2 M. Ranný, *Chem. Listy*, in press.
3 O. Cozzoli, *Riv. Ital. Sostanze Grasse*, 57 (1980) 136.
4 J. Newman, *Optimizing Reproducibility with Iastroscan TLC–FID Analyser*, *Fingerprinting of Heavy Oil by TLC–FID*, Instrument Application, Newman-Howells Assoc. Ltd., Winchester, Hants, 1981.
5 F. Bindler, P. Laugel and M. Hasselmann, *Deut. Lebensm.-Rundsch.*, 75 (1979) 111.
6 J. Poré, J. P. Houis and I. Rasori, *Rev. Fr. Corps. Gras*, 28 (1981) 111.
7 M. Zbirovský and V. Starý, *Sb. Vys. Sk. Chem.-Technol. Praze, Org. Chem. Technol.*, C28, submitted for publication.
8 J. C. Sipos and R. G. Ackman, *J. Chromatogr. Sci.*, 16 (1978) 443.
9 T. Fujii, R. Tanabe, K. Sasai and T. Tanaka, *Yukagaku*, in press.
10 R. G. Ackman, *Chem. Ind. (London)*, (1981) 715.
11 M. Ranný, J. Sedláček, E. Mareš, J. Svoboda and R. Seifert, *Seifen, Fette, Oele, Wachse*, submitted for publication.

Journal of Chromatography, 247 (1982) 335–339
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,074

Note

Rubidium benzenesulphonate —an efficient stationary phase for the separation of close-boiling organic isomers by gas chromatography

ASIT BHATTACHARJEE* and D. K. MUKHERJEE
Central Fuel Research Institute, P.O. F.R.I., Dhanbad, Bihar PIN-828108 (India)
and
DIPAK GUHA
Indian School of Mines, Dhanbad, Bihar, PIN-826004 (India)

(First received February 23rd, 1982; revised manuscript received May 26th, 1982)

The similarity of the physical and chemical properties of close-boiling aromatic and heterocyclic isomers poses serious problems in their separation. Techniques such as distillation, liquid chromatography, thin-layer chromatography and counter-current distribution frequently suffer from long analysis times, poor resolution and complexity of operation. Gas chromatography (GC) promises to be a fast, simple and efficient technique for this purpose and has been studied by various workers.

Various stationary phases have been used for the separation of close-boiling isomeric pairs such as *m*- and *p*-xylenes[1–3], *m*- and *p*-dichlorobenzenes[4,5], *m*- and *p*-tolualdehydes[6], *m*- and *p*-tolunitriles[7], 1- and 2-methylnaphthalenes[8], 3- and 4-methylpyridines[9–11], 2,4- and 2,5-dimethylpyridines[10,12], quinoline and isoquinoline[11,13], *m*- and *p*-toluidines[14], *m*- and *p*-cresols[15–17] and 2,4- and 2,5-xylenols[15,18]. However, no work appears to have been published on the GC separation of 3- and 4-cyanopyridines, 3- and 4-acetylpyridines, *m*- and *p*-dibromobenzenes or *m*- and *p*-bromobenzaldehydes. Even Bentone 34 (occasionally modified with a suitable liquid phase), which had been tried for the resolution of the largest number of isomeric pairs, was not universally satisfactory in terms of resolution, efficiency, peak symmetry, elution time, etc.

Rubidium benzenesulphonate (RBS) has been found to be an efficient stationary phase for the complete separation of 2,4- and 2,5-xylenols[19–21], an isomeric pair that had hitherto defied all attempts at a satisfactory separation by other workers. RBS was also found equally suitable for the separation of 2,4- and 2,5-dimethyl pyridines[19] and *m*- and *p*-cresols[19,21]. This paper considers the potential of RBS for the separation of other close-boiling isomers.

EXPERIMENTAL

The close-boiling isomeric pairs used in this study were chosen according to their availability. Whenever possible, pure chemicals were used; impure materials were purified by distillation or recrystallization. Two acidic, six basic and seven neutral isomeric pairs were studied (Table I).

0021-9673/82/0000–0000/$02.75

Experiments were carried out with a Perkin-Elmer 810 gas chromatograph fitted with a flame-ionization detector and a 1-mV Honeywell recorder. Stainless-steel columns (6 ft. × 1/8 in. O.D.) were used with nitrogen as the carrier gas (30 ml/min). The packing materials were prepared by slurrying the stationary phase, dissolved in a water–methanol mixture, with support, followed by removal of the solvent by heating.

Two columns were prepared as follows: A, 40% RBS + 2% Carbowax 20M + 2% ascorbic acid on Chromosorb P NAW (80–100 mesh); B, 40% RBS + 2% Carbowax 20M on Chromosorb P NAW (80–100 mesh). Column A was used for phenolic isomers and column B was used for the other isomers. Column A was activated at 175°C for 2 h under a flow of nitrogen. The temperature of activation was maintained below 181°C, as ascorbic acid decomposes above this temperature. Column B was similarly activated at 200°C for 3 h. The sample size was about 0.2 μl.

The separation factor (SF) was calculated using the following equation

$$\mathrm{SF} = \frac{d_{\mathrm{R_a}}}{d_{\mathrm{R_b}}} \qquad (1)$$

where, $d_{\mathrm{R_a}}$ and $d_{\mathrm{R_b}}$ are retention distances of components a and b, respectively (see Table I).

The general eqn. 2 for determining the resolution (R) was not found suitable for partially resolved peaks, particularly for studying the decrease in resolution.

$$R_{i,j} = 1.18 \cdot \frac{d_{\mathrm{R}_j} - d_{\mathrm{R}_i}}{W_{h/2,i} + W_{h/2,j}} \qquad (2)$$

where $W_{h/2,i}$ and $W_{h/2,j}$ denote peak width at half-height for compounds i and j, respectively. The following equation[22] was ultimately used throughout for determining the resolution:

$$R = \frac{f}{g} \cdot 100 \qquad (3)$$

where g is the length of the perpendicular drawn on the baseline through the lowest point of the trough between two resolved peaks up to the line joining the uppermost points of the two peaks, and f is the portion of the perpendicular from the lowest point of the trough to the point of intersection with the line joining the uppermost points of the two peaks. For two completely separated peaks the resolution is 100.

RESULTS AND DISCUSSION

Table I presents the resolutions and other characteristics for the isomeric pairs studied on the activated column. No universal and unique parameter could be identified that governs the retention behaviour of all of the isomeric pairs studied. Probably it is the concerted effect of ligand (lone electron pairs of the isomers)–Lewis acid ($\mathrm{Rb^+}$ ion) interactions, hydrogen bonding, dipole–dipole interactions, etc., with varying contributions that determine the elution characteristics. It is worth mentioning that the separation factor (2.02) and the extent of separation (Fig. 1) for quinoline and isoquinoline achieved in this work are probably the highest among those reported so far. The elution times of the completely separated pairs such as *m*- and *p*-dichlorobenzenes (Fig. 2) and *m*- and *p*-dibromobenzenes are also the shortest reported so far.

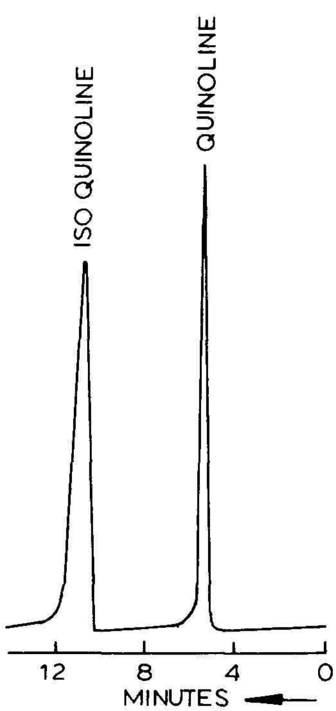
Activation plays a crucial role in enhancing the effectiveness of the stationary phase. It has been observed that significant resolution is possible only when the column is activated[19]. In this work, the decrease in resolution as a function of time, when an activated column was subjected to prolonged use, was studied. Necessarily,

TABLE I

RESOLUTIONS AND OTHER CHARACTERISTICS OF DIFFERENT ISOMERIC PAIRS

Isomers	*Boiling point (°C)*	*Column temperature (°C)*	*Column*	*SF*	*R (%)*	*Time of elution (min)*
(a) *p*-Xylene	138.0	80	B	0.79	88.5	11
(b) *m*-Xylene	139.0					
(a) 4-Methylpyridine	145.4	110	B	1.20	91.5	10
(b) 3-Methylpyridine	144.0					
(a) 2,4-Dimethylpyridine	158.0	130	B	1.30	97.5	6
(b) 2,5-Dimethylpyridine	157.0					
(a) *p*-Toluidine	200.5	130	B	1.21	94.0	27
(b) *m*-Toluidine	203.3					
(a) *p*-Tolualdehyde	204.0	145	B	1.17	87.2	18
(b) *m*-Tolualdehyde	199.0					
(a) 1-Methylnaphthalene	245.0	150	B	1.25	97.0	9
(b) 2-Methylnaphthalene	241.0					
(a) *p*-Dichlorobenzene	174.0	150	B	1.47	100.0	4
(b) *m*-Dichlorobenzene	172.0					
(a) *p*-Tolunitrile	217.0	150	B	0.83	97.0	17
(b) *m*-Tolunitrile	210.0					
(a) *p*-Dibromobenzene	219.0	170	B	1.27	100.0	5
(b) *m*-Dibromobenzene	219.5					
(a) *p*-Bromobenzaldehyde	Subl.	174	B	0.86	96.5	15
(b) *m*-Bromobenzaldehyde	233.0					
(a) 4-Acetylpyridine	214.0	175	B	0.80	96.5	11
(b) 3-Acetylpyridine	220.0					
(a) Isoquinoline	242.0	175	B	2.02	100.0*	12
(b) Quinoline	238.0					
(a) 4-Cyanopyridine	Subl.	180	B	0.80	97.0	8
(b) 3-Cyanopyridine	240.0					
(a) *p*-Cresol	201.0	150	A	1.21	95.0	29
(b) *m*-Cresol	202.0					
(a) 2,4-Xylenol	211.3	160	A	1.44	100*	14
(b) 2,5-Xylenol	211.5					

* Peaks are widely separated.



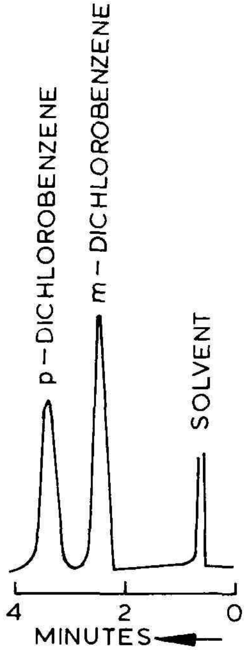


Fig. 1. Separation of quinoline and isoquinoline on column B at 175°C.

Fig. 2. Separation of *m*- and *p*-dichlorobenzenes on column B at 150°C.

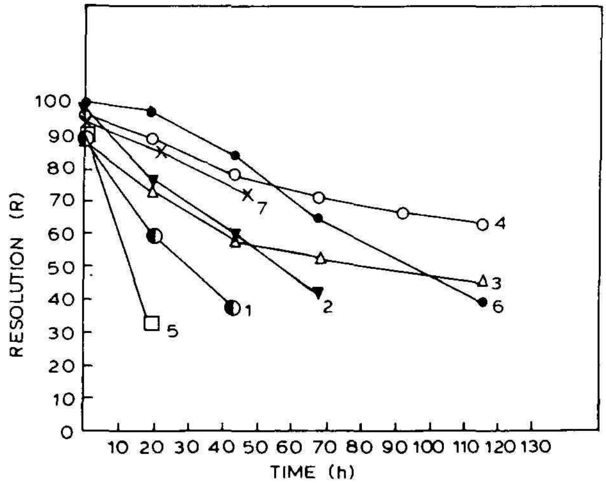


Fig. 3. Decrease in resolutions of seven isomeric pairs on columns A and B. 1 = *m*- and *p*-xylenes; 2 = 2,4- and 2,5-lutidines; 3 = *m*- and *p*-tolualdehydes; 4 = 1- and 2-methylnaphthalenes; 5 = *m*- and *p*-toluidines and 6 = *m*- and *p*-dichlorobenzenes on column B; 7 = *m*- and *p*-cresols on column A.

studies were conducted only on those isomeric pairs for which the column temperatures were low enough to preclude any *in situ* reactivation of the columns. Seven isomeric pairs were chosen for this purpose. Studies on the corresponding resolutions were determined at an interval of 20–24 h. Fig. 3 shows the gradual deactivation behaviour of columns A and B, activated (once only) at 175°C for 2 h and at 200°C for 3 h, respectively. Column deactivation and the decrease in resolution can be seen to be the fastest for *m*- and *p*-toluidines and the slowest for 1- and 2-methylnaphthalenes.

CONCLUSION

Fifteen close-boiling isomeric pairs were efficiently separated on a single stationary phase (RBS modified with Carbowax 20M, with or without ascorbic acid) probably for the first time. Some of the separations were highly satisfactory. Column deactivation studies were carried out in order to obtain an idea about the column life for specific isomeric pairs.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. P. N. Mukherjee and Mr. D. P. Dutta for their constant encouragement and helpful suggestions.

REFERENCES

1 J. V. Mortimer and P. L. Gent, *Nature (London)*, 197 (1963) 789.
2 A. C. Bhattacharyya and A. Bhattacharjee, *J. Chromatogr.*, 41 (1969) 446.
3 S. F. Spencer, *Anal. Chem.*, 35 (1963) 592.
4 J. V. Mortimer and P. L. Gent, *Anal. Chem.*, 36 (1964) 754.
5 Z. Witkiewicz and S. Popiel, *J. Chromatogr.*, 154 (1978) 60.
6 Y. Hoshika and G. Muto, *J. Chromatogr.*, 156 (1978) 346.
7 A. E. Habboush and K. A. B. Najm, *J. Chromatogr.*, 130 (1977) 161.
8 O. K. Guha and A. Bhaumik, *J. Chromatogr.*, 119 (1976) 181.
9 V. T. Brooks and G. A. Collins, *Chem. Ind. (London)*, (1956) 1021.
10 W. J. Kristen and R. G. G. Andren, *J. Chromatogr.*, 8 (1962) 531.
11 L. H. Klemm, J. Shabtai and F. H. W. Lee, *J. Chromatogr.*, 51 (1970) 433.
12 L. D. Gluzman, Yu. A. Slachinskii and V. P. Kostochka, *Coke Chem. U.S.S.R.*, 1 (1970) 40.
13 A. L. Brown and K. R. Buck, *Chem. Ind. (London)*, (1961) 714.
14 M. A. Hughes, D. White and A. L. Roberts, *Nature (London)*, 184 (1959) 1796.
15 V. T. Brooks, *Chem. Ind. (London)*, (1959) 1317.
16 A. Crespi and A. Galtieri, *Riv. Combust.*, 30 (1976) 82.
17 M. M. Anwar, C. Hanson and M. W. T. Pratt, *J. Chromatogr.*, 46 (1970) 200.
18 J. Kolšek and M. Matičič, *J. Chromatogr.*, 12 (1963) 305.
19 A. Bhattacharjee and A. Bhaumik, *J. Chromatogr.*, 115 (1975) 250.
20 A. Bhattacharjee and A. N. Basu, *J. Chromatogr.*, 71 (1972) 534.
21 A. Bhattacharjee and A. Bhaumik, *J. Chromatogr.*, 136 (1977) 328.
22 R. Kaiser, *Gas Phase Chromatography*, Vol. 1, Butterworths, London, 1963, p. 39.

Journal of Chromatography, 247 (1982) 340–341
Elsevier Scientific Publishing Company, Amsterdam -- Printed in The Netherlands

CHROM. 15,116

Note

Simple method for the removal of fines from even-sized column packing

J. R. RUNYON*
Central Research Plastics Laboratory, The Dow Chemical Company, Midland, MI 48640 (U.S.A.)
(Received June 16th, 1982)

In an attempt to clean up a batch of used controlled porosity glass (CPG, Electronucleonics) which had been screened to 44–53 μm size before use, we made a slurry of it in toluene (on the assumption that the contaminant was polymer). Then we attempted to remove the toluene on a sintered, ultrafine glass filter (Pyrex No. 36060) using a nitrogen pressure filter arrangement. The pores in this filter are nominally 0.9 to 1.4 μm so it was assumed that the toluene solution would pass easily and that the CPG would remain on the filter. However, the flow stopped after a few drops. Changing to a new "UF" filter produced the same results. We then tried a "coarse" filter without checking the pore size. The toluene "solution" ran through rapidly and was cloudy. Repeated slurrying and filtering through the same filter eventually gave a clear filtrate and clean-appearing CPG.

The nominal pore size of the "coarse" sintered glass filter is 40 to 60 μm, considerably larger than the particle size of the CPG. We deduced that the 44–53-μm particles must lodge in the tortuous path through the filter producing a filter bed of the same porosity as the chromatographic column itself. Anything that would pass through the column would pass through the filter bed. The "fine" frit had been plugged by something smaller than the screened packing.

Microscopic examination of the solids that settled out of the filtrate showed that they were, in fact, glass particles. A few of the glass particles were 44–53 μm in size, but mostly they were much smaller. The supernatant toluene solution was evaporated and the residue was polymer, as expected. It appeared that it had been broken pieces of the glass that had plugged the column end frits and the "fine" filter.

A fresh batch of screened CPG of the same size was slurried in acetone and filtered on a "coarse" filter. The filtrate again contained some glass pieces (mostly very small) and broken fragments, but a few were in the 44–53-μm range. The CPG on the funnel was dried by blowing nitrogen through it and then was dumped out and the funnel brushed to remove traces. Reversing the nitrogen flow blew out a cloud of CPG that was entirely in the 44–53 μm range. This packing evidently had lodged in the tortuous pathways through the sintered glass in spite of the fact that the nominal porosity of the sintered plate was considerably greater than the particle size. After

---

* Retired from The Dow Chemical Company. Present address: P.O. Box 2031, Midland, MI 48640, U.S.A.

0021-9673/82/0000–0000/$02.75

becoming lodged, this packing presented essentially the same porosity to migrating pieces (fines) as the column itself had presented.

The same technique was used to remove fines from 10-μm CPG (CPHGS, Electronucleonics) and from a sample of μStyragel (Waters Assoc.) with the same results. Microscopic examination of the packings before and after the cleanup showed that a very small fraction of fines initially present had been removed.

It would be expected that fines in a column would migrate to the end and if the end frit had pores of a size similar to or smaller than those fines, it would soon become plugged. On the other hand, a large pore end frit would allow fines to pass. In this case, the column would be cleared of fines before connecting the detector, or a second frit, in a "zero-volume" fitting that could be changed when it became plugged would follow the column. As an example, a 10 ft. × 2.2 mm I.D. column was closed at the outlet with a 45-μm stainless-steel frit and packed with CPGHS from a water slurry in an ultrasonic bath. When this column was put in operation, the first eluent was cloudy, and contained CPG particles from 10 μm down in size. After several column volumes had passed, the eluent became clear, and the column was put into use.

ACKNOWLEDGEMENT

The author is grateful to the Central Research Plastics Laboratory of The Dow Chemical Company for permission to publish this work.

Journal of Chromatography, 247 (1982) 342–346
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,073

Note

Alternating-current polarographic detection for reversed-phase ion-pair high-performance liquid chromatography of some benzoic acids

WŁODZIMIERZ KUTNER
Institute of Physical Chemistry of the Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw (Poland)

(First received March 19th, 1982; revised manuscript received May 27th, 1982)

Electrochemical detection has been successfully employed in reversed-phase ion-pair high-performance liquid chromatography (HPLC) in the separation of catecholamines and some other 3,4-dihydroxyphenylamines and carboxylic acids[1–4], with an ionic detergent, octylsulphate[1–3] or anions of strong inorganic acids and of trichloroacetic acid[4] as counter ions. Detection was performed with the aid of a flow-through thin-layer cell with a carbon paste sensor electrode. Because at carbon electrodes the differential capacity of the double layer at the electrode–solution interface cannot be measured reproducibly, only the faradaic response could be monitored in the amperometric mode of operation. Hence the determination was limited to redox substances only. However, using a dropping mercury electrode (DME) as the sensor electrode, non-faradaic signals can also be exploited and thus the presence of electroinactive substances that cause the change in the differential capacity can be detected in the column effluent[5–11]. Alternating-current (a.c.) polarography in both the total current[5–7,10,11] and phase-sensitive[8] modes and also alternating-voltage (a.v.) polarography[9,10] have been used for this purpose and it was possible to determine aromatic hydrocarbons[5–7], alkyl alcohols, alkyl carboxylic and sulphonic acids, different surfactants of the polyethylene glycol monoether type[8], cholanoic acids[9,10] and taurine conjugates of cholanoic acids[11].

In this paper we demonstrate the usefulness of a.c. polarographic detection in which the non-faradaic admittance is recorded for ion-pair HPLC. Benzoic, 4-hydroxybenzoic and acetylsalicylic acids, *i.e.*, compounds that are electroinactive but surface active on the DME, were chosen as model compounds. The separation conditions for these compounds in ion-pair HPLC with UV detection have been given elsewhere[12].

EXPERIMENTAL

Acetonitrile "for chromatography" (E. Merck, Darmstadt, G.F.R.) and doubly distilled water were used for the preparation of mobile phase solutions. Tetrabutylammonium hydroxide (TBAOH) was prepared from tetrabutylammonium iodide (E. Merck) according to a described procedure[12]. All other chemicals were of analytical-reagent grade.

Chromatographic experiments were performed using a Model 302 HPLC apparatus with a 5-μl injection valve and a type FTPD-101[13,14] flow-through polarographic detector (both made at the Institute of Physical Chemistry, Warsaw, Poland). The column used was a 250 × 4 mm I.D. stainless-steel column slurry packed[15] at 435 kg/m^2 with 10-μm LiChrosorb RP-18 (E. Merck) using tetrachloromethane–dioxan (1:1, v/v) as the suspending liquid. The flow-rate of the mobile phase was 1 ml/min.

A.c. polarographic recording was carried out with the use of a Radelkis OH-105 universal polarograph (Radelkis, Budapest, Hungary), operating at a frequency of 60 Hz and an amplitude of the alternating voltage, $\Delta E_{a.c}$, of 15 mV. The characteristics of the polarographic capillaries measured in the mobile phase solution were as follows in the steady-state a.c. polarographic experiments: m = 5.62 mg/sec, t_1 = 1.6 sec at $E = -0.1$ V *vs.* Ag/AgCl and h_{Hg} = 60 cm; and in HPLC experiments: m = 3.06 mg/sec, t_1 = 0.81 sec at $E = -0.1$ V *vs.* Ag/AgCl and h_{Hg} = 55 cm. The potential applied to the DME in the HPLC experiments was $E = -0.1$ V.

RESULTS AND DISCUSSION

A.c. polarographic detection of non-faradaic signals is possible only if a difference is observed between the curves of the double layer capacity *vs.* potential measured in blank solution and in the presence of a sample substance. Therefore, prior steady-state experiments are necessary for this purpose. For benzoic and 4-hydroxybenzoic acids adsorption studies at the DME only from aqueous solutions have been reported[16,17]. Therefore it was necessary to record a.c. polarographic curves for all of the sample acids in a solution of the composition of the mobile phase for ion-pair reversed-phase HPLC (Fig. 1). The most pronounced differences between

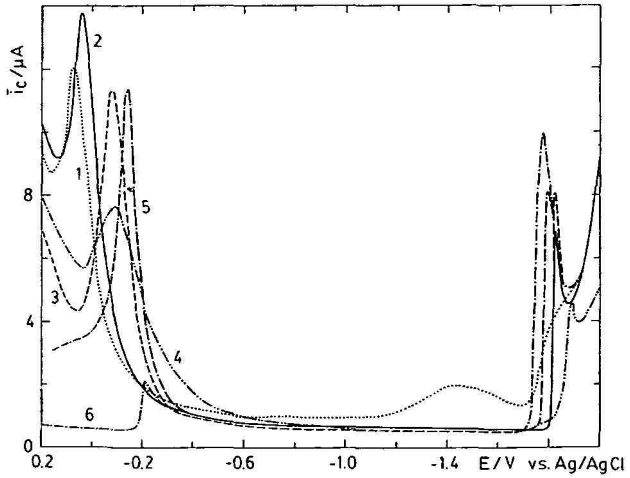


Fig. 1. A.c. polarographic mean capacity current, $\bar{i}_c$, as a function of potential for acetontrile–1/15 *M* phosphate buffer (pH 6) (20:80, v/v) (1), containing $2 \cdot 10^{-2}$ *M* TBAOH (2) and $2 \cdot 10^{-2}$ *M* benzoic acid (3), $2 \cdot 10^{-2}$ *M* acetylsalicylic acid (4), $5 \cdot 10^{-3}$ *M* 4-hydroxybenzoic acid (5) or $2 \cdot 10^{-2}$ *M* 4-hydroxybenzoic acid (6).

the curves of the mean capacity current, $\bar{i}_C$, *vs.* E recorded in the presence and in the absence of the acids are observed at a relatively positive potential range, which is why the potential, $E = -0.1$ V *vs.* Ag/AgCl, was further applied to DME in HPLC experiments (Fig. 2). The $\bar{i}_C$ *vs.* E curve for the blank solution is altered more by 4-hydroxybenzoic acid than by acetylsalicylic acid and least by benzoic acid. The elution of the acids proceeds in the same order (Fig. 2). This order, however, contrasts with the regularity observed, for instance, for homologous alkylbenzenes, for which the stronger the adsorption at the DME the greater is the retention time in a reversed-phase system[18]. In the multi-component system studied here a detailed discussion of the properties of the double layer at the DME is difficult and was not attempted because much more experimental data would be needed.

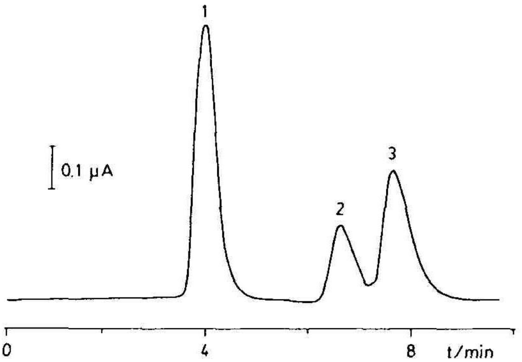


Fig. 2. HPLC results for 125 nmole of each of (1) 4-hydroxybenzoic acid, (2) acetylsalicylic acid, (3) benzoic acid with a.c. polarographic detection. $E = -0.1$ V *vs.* Ag–AgCl, $\Delta E_{a.c.} = 15$ mV; mobile phase, acetonitrile–1/15 *M* phosphate buffer (pH 6) (20:80, v/v)–0.02 *M* TBAOH; flow-rate, 1 ml/min; column, 250 × 4 mm I.D., LiChrosorb RP-18 (10 μm); sample size, 5 μl.

It is worth noting that in the mobile phase of pH 2 in the absence of TBAOH, *i.e.*, under conditions relevant to reversed-phase HPLC of the acids[12], alterations of the $\bar{i}_C$ *vs.* E curves by the acids were not large enough for a.c. polarographic detection to be applied, using a chosen potential common for all acids.

Tetrabutylammonium as the counter ion was added to the solution of the mobile phase in the form of TBAOH because other anions were inconvenient either for detection or for separation. For instance, perchlorates, which exhibited negligible adsorption at the DME, interfered strongly in ion-pair separations and peaks 2 and 3 in Fig. 2 were not separated. On the other hand, iodides, which have no effect on ion-pair separations, affected strongly the $\bar{i}_C$ *vs.* E curves and no effect of the acids studied could be measured. The tetrabutylammonium cation is strongly adsorbed at the DME in aqueous solutions at negative potentials[19–21]. It drastically changed the shape of the $\bar{i}_C$ *vs.* E curves in the mixed aqueous–methanol solution of the mobile phase at negative potentials and a sharp peak was observed (curve 2 in Fig. 1). However, at positive potentials the TBA cation caused no characteristic changes in the $\bar{i}_C$ *vs.* E curves.

Fig. 3 shows (a) calibration graphs of the peak surface area against the number of moles injected and (b) the logarithm of the peak surface area against the logarithm

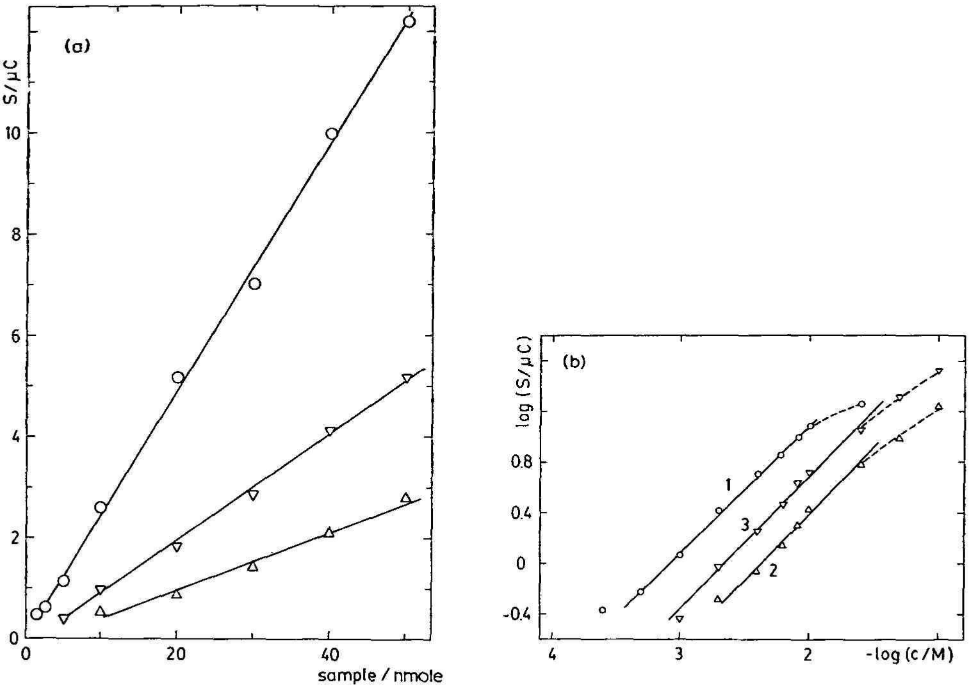


Fig. 3. Dependence of (a) peak surface area on amount of sample injected and (b) logarithm of peak surface area on logarithm of concentration, for (1) 4-hydroxybenzoic acid, (2) acetylsalicylic acid and (3) benzoic acid. Separation conditions as in Fig. 2.

of concentration over a wider concentration range. The response index of the detector, calculated for all acids from the slope of the curves in Fig. 3b, was close to unity (the mean value of the correlation coefficient was 0.997) over a concentration range of at least one order of magnitude. At higher concentrations deviations from linearity were observed (broken lines in Fig. 3b) owing to the non-linearity of the adsorption isotherms of acids at the DME. For concentrations of 4-hydroxybenzoic acid exceeding $4 \cdot 10^{-2}$ *M* the HPLC peak was split into two. This may be explained by the observed strong dependence of the adsorption peak on the $\bar{i}_c$ *vs*. *E* curve on the concentration of 4-hydroxybenzoic acid *i.e.* with an increase in the concentration of 4-hydroxybenzoic acid the adsorption peak decreases and shifts towards more negative potentials. At high concentrations the shape of the $\bar{i}_c$ *vs*. *E* curve is changed and a decrease in $\bar{i}_c$ is observed at positive potentials (curve 6 in Fig. 1). This may indicate phase transition of the adsorbate at the DME. Because of this complicated adsorption effect, careful potential control is essential for detection in HPLC experiments.

The sensitivities calculated from the intercept in Fig. 3a or the slope in Fig. 3b of the straight lines were 1240, 280 and 480 μC/mole for 4-hydroxybenzoic, acetylsalicylic and benzoic acid, respectively. The linear dynamic range extends for at least one order of magnitude and the detectability of 4-hydroxybenzoic acid is 0.625 nmole per 5-μl injection at a signal-to-noise ratio of 2.

In conclusion, it has been demonstrated that under carefully chosen conditions a.c. polarographic detection based on the changes in the double layer differential capacity is possible even in such a complicated system as reversed-phase ion-pair HPLC.

ACKNOWLEDGEMENTS

The author is grateful to Professor Göran Schill of the Biomedical Centre, University of Uppsala, Uppsala, Sweden, for the discussion that stimulated him to perform this work. Thanks are due to Ms. Victoria Santamaria of the University of Madrid, Madrid, Spain, for help with the experiments and to Dr. Barbara Behr of the Institute of Physical Chemistry, Warsaw, Poland, for critical comments. This work was carried out within research project 0.3.10 of the Polish Academy of Sciences.

REFERENCES

1 R. M. Riggin and P. T. Kissinger, *Anal. Chem.*, 49 (1977) 2109.
2 L. J. Felice, J. D. Felice and P. T. Kissinger, *Neurochemistry*, 31 (1978) 1461.
3 P. A. Asmus and C. R. Freed, *J. Chromatogr.*, 169 (1979) 303.
4 I. N. Mefford, M. Gilberg and J. D. Barchas, *Anal. Biochem.*, 104 (1980) 469.
5 W. Kemula, B. Behr, K. Chlebicka and D. Sybilska, *Rocz. Chem.*, 39 (1965) 1315.
6 W. Kemula, B. Behr, Z. Borkowska and J. Dojlido, *Collect. Czech. Chem. Commun.*, 30 (1965) 4950.
7 Z. Borkowska, D. Sybilska and B. Behr, *Rocz. Chem.*, 45 (1971) 269.
8 J. Lankelma and H. Poppe, *J. Chromatogr. Sci.*, 14 (14 (1976) 310.
9 W. Kemula and W. Kutner, *J. Chromatogr.*, 204 (1981) 131.
10 W. Kutner, B. Behr and W. Kemula, *Fresenius' Z. Anal. Chem.*, 312 (1982) 121.
11 A. Kutner, R. Jaworska, W. Kutner and A. Grzeszkiewicz, in S. Görög (Editor), *Advances in Steroid Analysis*, Akadémiai Kiadó, Budapest, 1982, p. 333.
12 A. Tilly-Melin, Y. Askemark, K.-G. Wahlund and G. Schill, *Anal. Chem.*, 51 (1979) 976.
13 W. Kutner, J. Dębowski and W. Kemula, *J. Chromatogr.*, 191 (1980) 47.
14 W. Kutner, J. Dębowski and W. Kemula, *Polish Pat.*, 119995, June 2, 1981 (Appl., P 210602, Oct. 30, 1978).
15 W. Daniewski, A. Bylina, A. Fiutkowski and A. Krupka, *Polish Pat.*, 112126, December 31, 1979 (Appl., P 197196, April 5, 1977).
16 K. M. Joshi and S. Rajagopalan, *Electrochim. Acta*, 17 (1972) 47.
17 J. Dojlido, M. Dmowska-Stańczyk and Z. Galus, *J. Electroanal. Chem.*, 94 (1978) 107, and refs. cited therein.
18 W. Kutner, *Report 15/1980*, Research Project 03.10 of the Polish Academy of Sciences, Warsaw, Poland.
19 A. N. Frumkin and B. B. Damaskin, *Dokl. Akad. Nauk SSSR*, 129 (1959) 862.
20 B. B. Damaskin and N. V. Nikolaeva-Fedorovich, *Zh. Fiz. Khim.*, 35 (1961) 1279.
21 S. G. Mairanovski and F. K. Ignateva, *Dokl. Akad. Nauk SSSR*, 241 (1978) 623.

Journal of Chromatography, 247 (1982) 347–351
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,075

Note

Gas chromatographic analysis of the vapours and gases discharged from the thermal fields of Kamchatka

V. B. BONDAREV*, N. V. PORSHNEV and D. F. NENAROKOV
Space Research Institute, Academy of Sciences of the U.S.S.R., Profsojusnaja 88, 117810 Moscow (U.S.S.R.)

(First received March 15th, 1982; revised manuscript received May 26th, 1982)

Studies into the composition of vapours and gases discharged during solfataric and fumarolic activity of volcanoes are of great interest not only for solving the problems of the geology, volcanology and utilization of thermal springs, but also for elucidating the abiogenic synthesis of various organic compounds.

Volcanic gaseous products contain hydrogen, water vapour, carbon monoxide, carbon dioxide, nitrogen, hydrogen cyanide, ammonia, hydrocarbons[1–5], and other components that can, under certain conditions, yield more complex substances as a result of reactions[6–8].

Reliable determination of the chemical composition of gases is complicated by a number of factors, the effect of which on the analysis results is difficult to assess. These include (1) difficulties in sampling, arising from elevated temperatures, dilution of the gases with air, etc., (2) variations in the composition of the sample during its storage and preparation for analysis and (3) changes in the composition of gas samples taken from a particular hydrothermal system in the course of time.

Although the analysis of volcanic gases by various methods has been widely covered in the literature, including tables summarizing worldwide data[9,10], the interpretation of most measurement results is difficult for the above reasons. This is particularly true for gases discharged by active volcanoes during eruption. The lack of satisfactory data concerning the composition of such volcanic gases has been mentioned elsewhere[6,11].

However, recent work published by Tazieff's group[12] shows the possibility of determining the chemical composition of volcanic gases at the eruption site with the aid of a field chromatograph. The analysis of gases *in situ* considerably enhances the reliability of measurements and allows the actual ratio of the components of mixtures to be determined at a particular point in time.

To solve these problems and to determine chromatographically the composition of gases at the point of sampling, a field chromatograph has been developed.

EXPERIMENTAL

Laboratory and field tests on the self-contained portable gas chromatograph have shown that its sensitivity threshold, in the case of calibration with air, is about

0021-9673/82/0000–0000/$02.75

10^{-5} g, the current through the bridge of the thermal conductivity cell (Gow-Mac, Madison, NJ, U.S.A.) being 150 mA. Calibration graphs were derived from peak areas. The samples were separated on stainless-steel columns (1.5 m × 2 mm I.D.) packed with Porapak Q (100–120 mesh) (Waters Assoc., Milford, MA, U.S.A.). The detector and columns were housed in a common thermostat which is essentially a steam-jacket. The measurements were carried out isothermally at ambient temperature and at the boiling point of the heat-transfer medium.

The total weight of the field chromatograph is 5.5 kg, including the bottle of carrier gas. The power supply to the instrument consists of 12-V silver–zinc batteries. The instrument was designed for 8 h of continuous operation. The electronic part of the instrument was illustrated and its applications described in an earlier paper[13].

The sampling of gases with a high water vapour content were carried out using procedures described elsewhere[1,14], without using any solutions absorbing individual components.

The gas samples from the Nizhne–Koshelevsky thermal field were taken in 1979 in 150–200 cm^3 glass ampoules that had been washed and evacuated in advance, which were immediately sealed. In 1980 gas samples were also taken in evacuated glass ampoules provided with an air-tight injector having a silicone-rubber septum. However, such ampoules were later found to be permeable to air when samples are stored for a long time in an evacuated state. The samples were analysed on Varian Aerograph 1800 and Pye 104 chromatographs equipped with CDS-101 and Kent Chromalog 2 integrators, then identified using an LKB-9000 gas chromatograph–mass spectrometer. The separations were carried out with 2 m × 2 mm I.D. glass columns packed with Porapak Q (60–80 mesh) and molecular sieve 5A (50–80 mesh) (Alltech, Arlington Heights, IL, U.S.A.) and 1 m × 2 mm I.D. columns packed with Tenax GC (30–60 mesh) (Applied Science Labs., State College, PA, U.S.A.).

RESULTS AND DISCUSSION

The results of field analyses showed that the major component in the vapours and gases discharged from the thermal fields after water vapour, is carbon dioxide (Table I). The concentration of methane does not exceed 1.5% by weight. The chro-

TABLE I

COMPOSITION OF GAS SAMPLES (WEIGHT-%)

Field analysis: August 1979.

Point of sampling (thermal fields)	*T(°C)*	$O_2 + Ar + N_2$	CO_2	H_2S	CH_4
Vostochnaya Pauzhetka well	97	40.9	57.1	N.D.*	2.0
Severo–Kambalny range 1st group, lower point	95	24.4	74.6	N.D.	1.0
Severo–Kambalny range, 1st group, upper point, vapour–gas spray	95	17.2	81.8	N.D.	1.0

* N.D. = Not detected.

matogram is shown in Fig. 1. Increasing the temperature to 100°C did not provide any additional information about the composition of the gases.

Our field experience has shown that the use of a portable self-contained chromatograph offers the advantages of statistical reliability of the results and the possibility of monitoring the sampling procedure, in view of the fact that the atmospheric oxygen contaminating the volcanic gas during sampling often results in changes to the chemical composition.

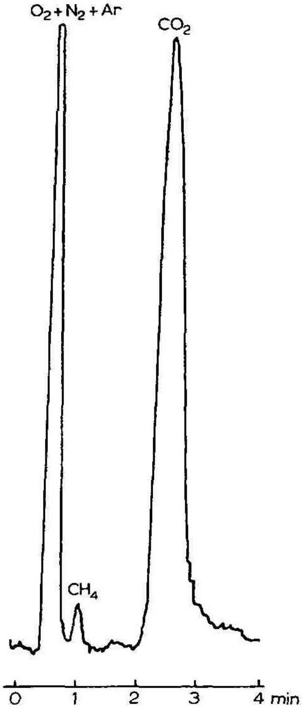


Fig. 1. Gas sample from Vostochnaya Pauzhetka well. Field chromatograph. Sorbent, Porapak Q; column, 1.5 m × 2 mm I.D.; sample volume, 300 μl; temperature of column and detector, 25°C; carrier gas, helium (30 cm^3/min).

However, an instrument designed for field application cannot incorporate many of the advances in gas chromatography. The concentrations of some gases are insignificant and lie below the sensitivity range of a field chromatograph. The separation of helium and hydrogen and of argon and oxygen requires low temperatures and adequately conditioned columns. This is why field work should preferably be complemented by laboratory analysis.

The use of a flame-ionization detector has made it possible to determine the concentration of light hydrocarbons (C_1–C_6). The results (Tables II and III) are presented with due account for the correction factors taken from Dietz's work[15]. The methane peak was selected as a reference as it is quantitatively present in all chromatograms. The gas taken from well 9 is characterized by a high content of nitrogen and methane with almost no water vapour. The argon to nitrogen ratio is almost twice as

TABLE II

COMPOSITION OF GAS SAMPLES (WEIGHT-%)

Laboratory analysis: August 1979.

Point of sampling (thermal fields)	$T(°C)$	$O_2 + Ar$	N_2	CO_2	H_2S	CH_4	C_2H_6	C_3H_8	ΣC_4H_{10}	ΣC_5H_{12}	ΣC_6H_{14}
Severo–Kambalny range, 1st group, lower point, spontaneous gas	15	1.24	5.76	92.64	N.D.*	0.34	$5.0 \cdot 10^{-3}$	$2.8 \cdot 10^{-3}$	N.D.	N.D.	N.D.
Verhne–Koshelevsky, vapour–gas spray	95	5.45	18.08	76.36	N.D.	0.11	$3.0 \cdot 10^{-3}$	$5.0 \cdot 10^{-4}$	N.D.	N.D.	N.D.
Nizhne–Koshelevsky well 9, depth 600 m	20	0.89	10.37	72.64	N.D.	14.63	1.13	0.48	0.20	0.05	0.20

* N.D. = not detected.

TABLE III

COMPOSITION OF GAS SAMPLES (WEIGHT-%)

Laboratory analysis: August 1980.

Point of sampling (thermal fields)	$T(°C)$	Ar	O_2	N_2	CO_2	H_2S	CH_4	C_2H_6	C_3H_8	ΣC_4H_{10}	ΣC_5H_{12}	ΣC_6H_{14}
Nizhne–Koshelevsky field:												
well 9, depth 600 m	12	0.41	1.94	14.00	66.64	N.D.*	15.95	0.73	0.24	$6.0 \cdot 10^{-2}$	$< 10^{-2}$	N.D.
		0.49	2.12	14.96	64.80	N.D.	16.63	0.74	0.20	$5.0 \cdot 10^{-2}$	$< 10^{-2}$	N.D.
		0.54	1.54	13.12	68.50	N.D.	15.18	0.77	0.23	$6.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$	N.D.
		0.35	1.37	11.62	67.84	N.D.	17.85	0.74	0.20	$3.0 \cdot 10^{-2}$	$< 10^{-2}$	N.D.
well 10, sample 1020	12	0.21	1.60	6.31	86.37	1.34	3.93	0.14	$4.0 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$	

* N.D. = Not detected.

high as that in air. It has also been established that the condensates of the samples taken in the Koshelevsky thermal fields contain C_9–C_{14} hydrocarbons, aromatic hydrocarbons, aldehydes, etc.[4]. The results (Tables II and III) and published data[5,16] suggest that the chemical composition of the gases from the Nizhne-Koshelevsky thermal field has remained stable over several decades. The gas from well 10 was sampled during drilling and provides no additional data except for having a unique composition.

ACKNOWLEDGEMENTS

Thanks are due to Yu. A. Taran and V. P. Pilipenko, research workers at the Institute of Volcanology, Far Eastern Scientific Centre, U.S.S.R. Academy of Sciences, for their generous help in our experiments.

REFERENCES

1 L. A. Basharina, *Byull. Vulkanol. Stn. Akad. Nauk SSSR*, 24 (1956) 21.
2 A. M. Chirkov, L. N. Barabanov, L. A. Basharina and K. K. Zelenov, *Byull. Vulkanol. Stn. Akad. Nauk SSSR*, 48 (1972) 33.
3 S. I. Naboko (Editor), *Volcanism, Hydrothermal processes and Ore-Bearing*, Nedra, Moscow, 1974, p. 209.
4 L. M. Mukhin, V. B. Bondarev and E. N. Safonova, *Mod. Geol.*, 6 (1978) 119.
5 V. V. Ivanov, *Tr. Lab. Vulkanol. Akad. Nauk SSSR*, 12 (1956) 197.
6 S. W. Fox and K. Dose, *Molecular Evolution and the Origin of Life*, Freeman, San Francisco, 1972.
7 V. B. Bondarev and N. V. Porshnev, *Dokl. Akad. Nauk SSSR*, 252 (1980) 455.
8 J. Oro and J. Han, *J. Gas Chromatogr.*, 9 (1967) 480.
9 D. E. White and G. A. Waring, in M. Fleischer (Editor), *Data of Geochemistry*, 6th ed., *U.S. Geol. Surv. Prof. Pap.*, No. 440-K, 1963, p. 1.
10 G. E. Sigvaldason, in *Phys. Volcanol.*, Amsterdam, 1974, p. 215.
11 V. N. Sharapov, I. B. Simbirev, G. A. Tretyakov, L. B. Milova and A. I. Vasileva, *Magmatism and Hydrothermal Systems of the Volcano Mutnovsky, Kamchatka*, Nauka, Novosibirsk, 1979, p. 100.
12 Le Guern, P. Bicocchi, A. Nohl and H. Tazieff, *C.R. Acad. Sci.*, 288 (1979) 867.
13 D. F. Nenarokov, S. S. Karavaev, V. V. Brazhnikov and K. I. Sakodynskii, *Chromatographia*, 4 (1971) 106.
14 A. M. Pozhkov, R. V. Guseva and A. A. Ponomarev, in *Hydrothermal Processes in Regions of Contemporaneous Magmatism Activity*, Nauka, Moscow, 1977, p. 198.
15 W. A. Dietz, *J. Gas Chromatogr.*, 5 (1967) 68.
16 N. S. Beskrovny and V. A. Lobkov, in *Hydrothermal Processes in Regions of Contemporaneous Magmatism Activity*, Nauka, Moscow, 1977, p. 110.

Journal of Chromatography, 247 (1982) 352–355
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CHROM. 15,069

Note

Gas–liquid chromatographic assay of polycyclic aromatic hydrocarbon mixtures: specifically modified method for rat tissues

R. MODICA, M. FIUME and I. BARTOŠEK*
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)
(First received April 28th, 1982; revised manuscript received May 25th, 1982)

The metabolism of polycyclic aromatic hydrocarbons (PAHs) and mechanisms through which they induce tumours in laboratory mice and rats following administration of single compounds have been widely studied. However, little is known about the distribution, availability, elimination and interactions of PAHs when a mixture is administered. Until only a few years ago there were technical limitations to this approach: the few reported methods involved too many steps; the analyses were therefore time consuming and failed to achieve acceptable separations of some compounds. Dao *et al.*[1] described a fluorimetric method for the determination of some PAHs in various organs of rats; this method, however, could not be applied to PAH mixtures because it required the preliminary separation of all of the individual compounds. Analogous studies were reported by Flesher[2] on [^{3}H]7,12-dimethylbenzanthracene (DMBA) and by Kotin *et al.*[3] and Schlede *et al.*[4] on [^{14}C]-3,4-benzopyrene. As these methods required different isotopes for to labelling each polycyclic hydrocarbon and did not discriminate between radioactivity bound to the parent compound and its metabolites, they are difficult to use for the analysis of PAH mixtures.

Gammal *et al.*[5] applied a combined method [thin-layer chromatography + gas–liquid chromatography (GLC)] in a study of distribution of 7,12-DMBA in some organs of rats, but the separation of the isomers was not possible. Howard *et al.*[6], McGinnis and Norris[7] and Grimmer and Hildebrandt[8] developed procedures for determining PAH mixtures in smoked foods, high-protein foods, oils, fats and yeasts; because of the high specificity and sensitivity required for such studies, the methods involved many purification steps and very lengthy procedures.

Subsequently, Santoro *et al.*[9] developed a method for the determination of 11 PAHs in yeasts, involving a relatively rapid analyses and giving high sensitivity. This present paper describes modifications to this method intended to simplify extraction and to accelerate the assay of PAHs in different rat tissues for kinetic studies.

EXPERIMENTAL

Chemicals

Triphenylene and benzo[*e*]pyrene were supplied by Aldrich (Beersee, Belgium), chrysene by EGA Chemie (Steinheim, G.F.R.) and benz[*a*]anthracene by Fluka (Buchs, Switzerland). All PAHs were of 99% purity.

0021-9673/82/0000–0000/$02.75

N,N′-Bis(*p*-phenylbenzylidene)-α,α'-bi-*p*-toluidine (BPhBT) was prepared by condensation of toluidine and *p*-phenylbenzaldehyde as described in previous papers[9,10]. Silica gel 60 (70–230 mesh ASTM) was activated for 3 h in an oven at 120°C, then water was added (15%) with shaking. The silica gel was stored in a stoppered flask at room temperature for about 2 h in order to allow the water to distribute evenly. Alumina (Merck, 90 Aktiv) was prepared as described[9].

All other reagents were of analytical-reagent grade and the water was doubly distilled.

Animals

Tissues obtained from CD-COBS rats (Charles River, Italy) were utilized. Blood was collected in a test-tube containing 10% (v/v) of heparin (2 I.U.). Mammary and parametrial adipose tissue, liver and brain were rapidly excised and frozen on solid carbon dioxide. All samples were stored at −20°C until taken for analysis.

Apparatus

A Model 2450 gas chromatograph (Carlo Erba, Milan, Italy) with a flame-ionization detector and a column (1.20 m × 3 mm I.D.) packed with 2.5% (w/w) BPhBT on Chromosorb W 100 were used; deteails of the column preparation and conditions for the GLC assay were the same as previously described[9,10].

Initial preparation of samples for PAH assay

After thawing, blood was diluted with 10 ml of doubly distilled water; brain and liver were homogenized in 5 volumes of water (for liver homogenate only, an amount corresponding to 2.5 g of the organ was used for PAH analysis).

Mammary and adipose tissue were hydrolysed [ethanol (10 volumes) + water (3 volumes) + potassium hydroxide (1 g per g of tissue)] for 1 h under reflux at 80°C on a hot-plate with a magnetic stirrer.

Extraction

All samples were extracted three times with 10 ml of cyclohexane (at 50°C, shaking for 1 min, centrifugation for 5 min at 2500 *g*). The extracts of hydrolyzed adipose tissue were further washed twice with water (10 ml) and dried with Na_2SO_4.

Purification

For blood, brain, liver and mammary tissues, the extracts were concentrated to 0.3–0.4 ml under a flow of nitrogen in a water-bath at 40°C, then purified by chromatography on alumina [5 g with 10% (w/w) of water per sample] in a column (1 cm I.D.) with a fritted disc and a PTFE stopcock; no pre-washing of the column was needed. The concentrate and the following washes of the 50-ml test-tubes (four portions of 0.3–0.4 ml of cyclohexane) were applied with a glass pipette on the top of the column. The PAHs were eluted with cyclohexane (10 ml). For the extracts of adipose tissue purification by chromatography on alumina [5 g with 10% (w/w) of water) in a column of 1.3 cm I.D., no pre-washing of the columns was needed.

The extracts and cyclohexane washings of the 50-ml test-tubes were eluted, cyclohexane (10 ml) was added to the column and the eluate was collected in a 50-ml test-tube, and concentrated to 0.3–0.4 ml under a stream of nitrogen in a water-bath at 40°C.

A further purification step by chromatography on silica gel[9] was always required for the samples of adipose tissue.

GLC analysis

The packing material [2.5 % (w/w) of N,N′-bis(*p*-phenylbenzylidene)-α,α′-bi-*p*-toluidine on 100–120 mesh Chromosorb W HP] was prepared by the solvent slurry method, fluidized, dried with nitrogen and resieved to 100–120 mesh. The column was conditioned overnight at 290°C with a flow of carrier gas (nitrogen) at 8 ml/min. The oven temperature was 265°C, injector temperature 275°C, air flow-rate 300 ml/ml, hydrogen flow-rate 22 ml/min and carrier gas flow-rate 30 ml/min. An internal standard [benzo(*e*)pyrene] was added to the alumina or silica gel eluate and the sample was concentrated to about 100 μl under a stream of nitrogen stream at 40°C, thoroughly washing the test-tube walls; 1–2 μl of the concentrate were injected into the gas chromatograph. The correction factor for each PAH was calculated using the internal standard as described previously[9].

RESULTS AND DISCUSSION

The modifications introduced for the GLC determination of PAHs in rat tissues shorten the method of Santoro *et al.*[9] and permit the rapid analysis of many different biological samples. About 60–100 analyses of tissue PAHs can be performed by two technicians each week. The time saving in the performance did not result in a decrease in specificity and sensitivity. As indicated in Table I, the recovery was satisfactory, ranging from a minimum of 83 % to a maximum of 95 %. The sensitivity limits were 0.05 μg for the three isomers. The reproducibility of the results was particularly good as indicated by the low standard errors reported in Table I. The coefficient of variation ranged between 1 and 5 %.

The application of this method to the determination of the kinetic parameters of PaHs administered singly or in mixtures to rats will be reported elsewhere.

TABLE I

RECOVERY OF POLYCYCLIC AROMATIC HYDROCARBON MIXTURES

10 μg of each hydrocarbon were added per gram of rat tissue. Results are means ± standard errors of 5 experiments.

Tissue	*Triphenylene (μg)*	*Chrysene (μg)*	*Benz(a)anthracene (μg)*
Blood	9.551 ± 0.091	9.541 ± 0.012	9.285 ± 0.073
Liver	8.589 ± 0.131	8.390 ± 0.130	8.342 ± 0.121
Brain	9.187 ± 0.142	8.756 ± 0.288	8.729 ± 0.229
Mammary tissue	9.355 ± 0.175	9.344 ± 0.263	9.313 ± 0.146
Parametrial adipose tissue	8.892 ± 0.135	9.478 ± 0.441	8.995 ± 0.127

ACKNOWLEDGEMENTS

This study was supported by a contract from the Italian National Research Council in the framework of the project "Controllo della crescita neoplastica" (CNR Contract 80.01476.96). A generous contribution from the Italian Association for Cancer Research, Milan, Italy, is gratefully acknowledged.

REFERENCES

1 T. L. Dao, F. G. Bock and S. Crouch, *Proc. Soc. Exp. Biol. Med.*, 102 (1959) 635.
2 J. W. Flesher, *Biochem. Pharmacol.*, 16 (1967) 1821.
3 P. Kotin, H. L. Falk and R. Busser, *J. Nat. Cancer Inst.*, 23 (1959) 541.
4 E. Schlede, R. Kuntzman, S. Haber and A. H. Conney, *Cancer Res.*, 30 (1970) 2893.
5 E. B. Gammal, K. K. Carroll, B. H. Muhlstock and E. R. Plunkett, *Proc. Soc. Exp. Biol. Med.*, 119 (1965) 1086.
6 J. W. Howard, R. T. Teague, Jr., and R. H. White, *J. Ass. Offic. Anal. Chem.*, 49 (1966) 595.
7 E. L. McGinnis and M. S. Norris, *J. Agr. Food Chem.*, 23 (1975) 221.
8 G. Grimmer and A. Hildebrandt, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 631.
9 A. Santoro, R. Modica, S. Paglialunga and I. Bartošek, *Toxicol. Lett.*, 3 (1979) 85.
10 G. N. Janini, G. M. Muschik, J. A. Schroer and W. L. Zielinski, Jr., *Anal. Chem.*, 48 (1976) 1879.

Journal of Chromatography, 247 (1982) 356–358
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,072

Note

Analysis of essential oil mixtures in ointments

KARL-ARTUR KOVAR* and ALBRECHT SAKMANN
Pharmazeutisches Institut der Universität Tübingen, Auf der Morgenstelle 8, D-7400 Tübingen (G.F.R.)

(First received April 28th, 1982; revised manuscript received May 28th, 1982)

Essential oils are useful medicaments for the treatment of various diseases and are applied as antirheumatics, expectorants, carminatives and anti-inflammatory agents. Several essential oils are frequently mixed together in ointments, liniments and liquors. In order to establish the quality of such commercial preparations, it is necessary to determine the mixing ratio of particular essential oils. Previous quantitative analyses of essential oil mixtures by high-performance liquid chromatography (HPLC) in comparison with gas chromatography (GC)[1] showed that both methods are suitable but should be applied in different situations. For essential oil mixtures that contain strong UV-absorbing constituents, HPLC is preferred as it produces more complete resolution and is faster. On the other hand, GC is to be preferred for those mixtures which contain non-specifically detectable compounds.

In this work, these elementary findings were applied to the analysis of essential oil mixtures in ointments. A complex composite unguent containing both oxygenated and oxygen-free terpenes, *e.g.* pine needle, eucalyptus, mint and melissa oil, in addition to camphor and menthol was selected as a model. The principle of the proposed method is based on the separation of the terpene compounds from the ointment base by steam distillation using *n*-hexane to take up the volatile compounds. The diluted *n*-hexane solution was investigated by GC and the composition and mixing ratio of the essential oils were determined via their terpene contents using fenchone[2] as an internal standard.

EXPERIMENTAL

Steam distillation

Steam distillation was carried out in a special apparatus for the determination of essential oils in vegetable drugs as described in the European Pharmacopoeia (Ph.Eur.III)[3]. An accurately weighed 5.0-g sample of unguent was introduced into the distillation flask and 50.0 mg of fenchone and 300 ml of water were added. Then the condenser apparatus was attached and the distillation rate was adjusted to 2–3 ml/min. The distillate was collected in the graduated tube, using 1.00 ml of *n*-hexane to take up the volatile compounds. After 2 h the volume of the *n*-hexane solution in the graduated tube was read and the volume of a blank carried out under the same conditions using 5.0 g of ointment base was substracted (see the Ph.Eur.III[3]). The difference represents the amount of essential oil mixture and terpenes in the weight of ointment taken. The result was calculated as millilitres of oil per 100.0 g of ointment.

0021-9673/82/0000–0000/$02.75

Gas chromatography

A Hewlett-Packard Model 5711A gas chromatograph equipped with hydrogen flame-ionization detector (FID) and a Model 5671 A, automatic sample injector, was used. GC columns (4.2 m × 0.3 cm I.D.) were packed with 10% Carbowax 20M on Chromosorb W AW DMCS (60–80 mesh). The temperature of the injector and detector was 250°C and the column oven was heated to 80°C. Four minutes after injection the temperature was programmed at a rate of 4°C/min to 150°C, at which temperature it was maintained for 8 min. The carrier gas (nitrogen) flow-rate was 25 ml/min. A 2.0-μl volume of the collected distillate, diluted with *n*-hexane (1:10) and dried over anhydrous sodium sulphate, was injected. Peak areas were determined with a Hewlett-Packard Model 3370 integrator. Terpene contents were calculated using fenchone as an internal standard. Melissa oil was determined via citronellal and limonene, mint oil via menthone, eucalyptus oil via cineol and pine needle oil via peak 1 (a mixture of several monoterpene hydrocarbons).

RESULTS AND DISCUSSION

GC was preferred to HPLC because stability studies had to be carried out under constant conditions over a long period, and because HPLC columns decrease in efficiency much faster than GC columns. Isolation of the essential oils including camphor and menthol from the ointment was essential in order to avoid carbonization of the dissolved ointment base in the injector, contamination of the column and interference with peaks of the oxygen-free terpenes in the chromatogram.

Steam distillation carried out in the special apparatus described in the Ph.Eur.III[3] was effective. Under these conditions the volatile constituents of the ointment base (0.23 ml per 100.0 g ≈ 1.86% of the total mixture) did not interfere in the analysis of the terpenes as they were eluted later. Contrary to the method of the Ph.Eur.III[3], *n*-hexane was used instead of xylene to collect the volatile compounds, otherwise the determination of the percentage content of pine needle oil would have been difficult. Loss of *n*-hexane was not disadvantageous as the internal standard fenchone had been added to the distillation flask before steam distillation took place.

On analysis of an ointment sample containing 11.5 g of essential oils and terpenes per 100.0 g (≈ 12.36 ml per 100.0 g; $d_{20}^{20} = 0.9022$), an average value of $\bar{x} = 12.02$ ml per 100.0 g ($n = 10$) using ointment base as a blank was obtained; this corresponds to a recovery of 97.2%.

The GC conditions proposed by Kovar and Friess[1] were slightly modified by using columns loaded with more Carbowax (10%); thus a higher effective plate number (>4200) was achieved in order to improve the resolution of the critical pair limonene–cineol and to facilitate the quantification of pine needle oil. The content and recovery of each essential oil were determined via characteristic components (Fig. 1, Table I), which had been selected before preparing the unguent. Each of the related terpenes in the distillate was identified by mass spectrometry. The peaks of camphor (7), cineol (3), menthol (8) and pine neddle oil (1) were well separated and quantified. The determination of mint oil via menthone (5), however, was complicated by incomplete separation from citronellal (6).

Quantification of melissa oil based on measuring geraniol (9) did not give satisfactory results and other constituents, *e.g.*, citronellal (6) and limonene (2), were therefore employed. Citronellal showed a better recovery but also a higher standard deviation than limonene. Accordingly, citronellal was used to measure the amount of melissa oil in in the mixture, whereas limonene served in stability studies in order to

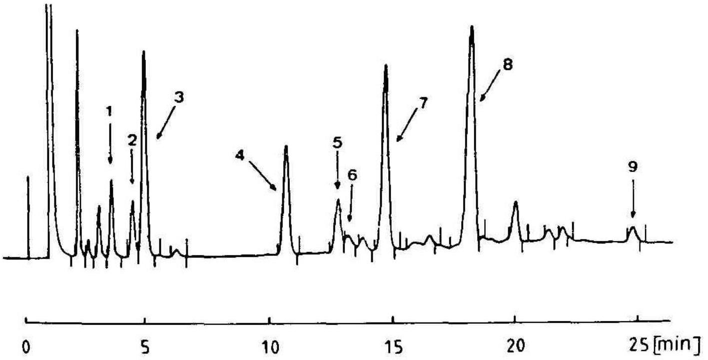


Fig. 1. GC separation of a mixture of essential oils and terpenes in ointment after steam distillation. Peaks: 1 = pine needle oil; 2 = limonene; 3 = cineol; 4 = fenchone (internal standard); 5 = menthone; 6 = citronellal; 7 = camphor; 8 = menthol; 9 = geraniol.

register any small loss of its content. The recoveries of the individual essential oils and of camphor and menthol in mixtures were found to be between 98.9 and 101.9%, the relative standard deviations ranging from 0.8 to 2.16% (Table I). Thus, the proposed method proved to be highly satisfactory for the analysis of mixtures of essential oils and terpenes. In principle, it can also be applied to other mixtures of different ratios and compositions.

TABLE I

RECOVERY OF ESSENTIAL OILS AND TERPENES FROM THE PREPARED OINTMENT (n = 10)

Essential oils and terpenes	*Peak No. in Fig. 1 for determination*	*Content (g per 100.0 g of ointment)*		*Standard deviation (g per 100.0 g of ointment)*	*Relative standard deviation (%)*	*Recovery (%)*
		Calculated	*Found*			
Pine needle oil	1	1.100	1.103	0.017	1.58	100.3
Eucalyptus oil	3	2.210	2.184	0.043	1.99	98.8
Mint oil	5	1.970	1.973	0.028	1.43	100.1
Melissa oil	6	1.870	1.905	0.041	2.16	101.9
Limonene	2	1.870	2.271	0.021	0.94	121.5
Camphor	7	2.000	2.006	0.016	0.80	100.3
Menthol	8	2.808	2.800	0.051	1.82	99.7

Stress tests at 41, 51 and 61 C over a period of 12 weeks were carried out under the conditions proposed by Grimm and Schepky[4]. Significant deviations from the original contents of each of the essential oils were not observed; this result agrees with those of Neuwald and Scheel[5]. It is concluded that the essential oils and terpenes used in the present work are stable towards autoxidation for more than 5 years.

REFERENCES

1 K.-A. Kovar and D. Friess, *Arch. Pharm. (Weinheim)*, 313 (1980) 416.
2 H. Glasl and H. Wagner, *Deutsch. Apoth.-Ztg.*, 114 (1974) 146 and 363.
3 *European Pharmacopoeia*, Vol. III, Maisonneuve S.A., 57 Sainte-Ruffine, France, 1975, p. 68.
4 W. Grimm and G. Schepky, *Stabilitätsprüfungen in der Pharmazie*, Editio Cantor, Aulendorf, 1980.
5 F. Neuwald and D. Scheel, *Pharm. Ind.*, 31 (1969) 879.

Journal of Chromatography, 247 (1982) 359–365
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,068

Note

Hochleistungs-Flüssigkeits-Chromatographische Analyse von Digitalis-Blattextrakten

II. Quantitative Analyse

M. WICHTL*, W. WICHTL-BLEIER und M. MANGKUDIDJOJO
Institut für Pharmazeutische Biologie der Philipps-Universität, Deutschhausstrasse 17¹/₂, D-3550 Marburg/L. (B.R.D.)

(Eingegangen am 27. April 1982; geänderte Fassung eingegangen am 25. Mai 1982)

In einer ersten Mitteilung[1] haben wir über die qualitative Analyse von Digitalis-Blattextrakten mittels Hochleistungs-Flüssigkeits-Chromatographie (HPLC) berichtet. Wie dort bereits angekündigt wollen wir nun die Bedingungen für die quantitative Analyse mitteilen.

Um Schwankungen in der Volumendosierung bei der Probeneingabe auszuschalten haben wir für die quantitative Bestimmung einen internen Standard gewählt. Nach zahlreichen Vorversuchen haben wir bei isokratischem Betrieb mit Acetonitril–Wasser (30:70) Hexobarbital (1,5-Dimethyl-6-[1′-cyclohexenyl]-barbitursäure), sowie bei der Gradientenelution Diazepam (7-Chlor-1-methyl-2-oxo-3H-1,4-benzodiazepin) als besonders geeignet befunden. Beide Substanzen weisen in den verwendeten Fliessmitteln ein Absorptionsmaximum von 225 nm auf und interferieren praktisch nicht mit einem der Digitalisglykoside. Während Diazepam universell anwendbar ist, kann bei der Benutzung von Hexobarbital unter bestimmten Bedingungen ein Überlappen des peaks von Glucoevatromonosid mit dem des Hexobarbitals beobachtet werden. Da die meisten Cardenolidglykoside in Acetonitril–Wasser (35:65) ein Absorptionsmaximum bei 222–226 nm aufweisen (in methanolischer Lösung liegt es bei 218–219 nm) haben wir als Messwellenlänge bei der quantitativen Bestimmung durchwegs 225 nm gewählt.

Zunächst haben wir mit 11 Digitalisglykosiden, die uns in ausreichender Menge als analysenreine Substanzen zur Verfügung standen eine Calibrierung vorgenommen und hierauf unter konstanten Bedingungen mit Glykosidgemischen, die mengenmässig unterschiedlich zusammengesetzt waren, Calibrierungsläufe mehrfach wiederholt, wobei die Wiederfindungsraten zwischen 94.5 und 99.1 % lagen. Unter Zugrundelegung der bei Gradientenbetrieb ansteigenden Basislinie haben wir das Rechenprogramm des Gerätes so festgelegt, dass die einzelnen Zählwerte (counts) unter Berücksichtigung der Calibrierung und des inneren Standards direkt in mg/100 g Blattdroge umgerechnet werden. Da das Gerät auch die counts jedes einzelnen peaks ausdruckt, ist immer eine zusätzliche Kontrolle möglich.

EXPERIMENTELLES

Gerät und Analysendaten

Wir verwendeten einen 1084 B Hewlett-Packard Flüssigkeits-Chromatograph, und LiChrosorb RP-8 (10 μm) und RP-18 (10 μm) Säulen, Länge 20 cm × 4.6 mm I.D., Stahl. Mobile Phase: Wasser–Acetonitril, Gradientenelution von (73:27) nach (61:39) innerhalb 35 min, linearer Anstieg. Temperatur: 40°C. Durchfluss: 1.5 ml/min. Variabler Wellenlängendetektor: Hewlett-Packard 1030 B. Schreiber–Integrator: LC-Terminal 79850 B Hewlett-Packard. Injizierte Probenmenge: 10–30 μl, entsprechend *ca.* 2.7–8.1 mg Blattdroge.

Reagentien

Lösungsmittel: LiChrosolv (Merck, Darmstadt, B.R.D.). Chemikalien z. Analyse (Merck). Die für Vergleichszwecke verwendeten herzwirksamen Glykoside haben wir von den Firmen Boehringer (Mannheim, B.R.D.) und Sandoz (Basel, Schweiz) erhalten.

Probenvorbereitung

Feingepulverte Digitalis-Blattdroge (1.500 g) werden in einem tarierten Rundkolben mit 15.0 g heissem Ethanol (70 %) übergossen und 15 min unter Rückflusskühlung auf dem Wasserbad erhitzt. Nach Abkühlen auf Raumtemperatur fügt man 25.0 g Wasser und 10.0 g einer Lösung von 15 g $Pb(CH_3COO)_2 \cdot 2H_2O$ in 100 ml Wasser zu, ergänzt mit Wasser auf 60.00 g und mischt gut durch. Hierauf wird der entstandene Niederschlag abzentrifugiert; von der klaren überstehenden Lösung versetzt man 50.0 g mit 12 g einer Lösung von 10 g $Na_2HPO_4 \cdot 2H_2O$ in 100 ml Wasser, mischt gut durch und zentrifugiert. Von der überstehenden klaren Lösung werden 57.0 g (entspr. 1.15 g Digitalis-Blattdroge) mit 1 × 30 und 3 × 20 ml Chloroform–Isopropanol (3:2) ausgeschüttelt. Die vereinigten organischen Phasen bringt man unter vermindertem Druck bei maximal 40° C zur Trockne (Rotationsverdampfer), löst den Rückstand in 3.00 ml Methanol p.a. und setzt 1.00 ml einer frisch bereiteten Lösung von 40.00 mg Diazepam/100 ml Methanol p.a. (entspr. 0.4 mg Diazepam) zu. Die Lösung wird über Millipore filtriert; je nach Digitalis-Art werden für die HPLC-Analyse 10, 20 oder 30 μl verwendet. Die Menge des internen Standards kann bei Bedarf auch reduziert werden.

Messung

Um sich über die Trennung der Glykoside und den Verlauf der Basislinie einen Überblick zu verschaffen wird zunächst ein Testchromatogramm angefertigt. Hierauf gibt man dem Rechner die aus dem Eichlauf (calibration run) errechneten Faktoren [Menge/Peakfläche; amount/area], die zu erwartenden Retentionszeiten sowie die sich aus dem Testlauf ergebende Integrationsart bei nicht vollständig getrennten peaks (Tangentenverfahren, bzw. Lot zur Basislinie) ein. Die peaks von nicht calibrierten Substanzen wurden als Digitoxin berrechnet.

ERGEBNISSE UND DISKUSSION

Quantitative HPLC-Analyse

In Fig. 1 wird das für die Calibrierung von 11 reinen Digitalisglykosiden ermittelte Chromatogramm dargestellt. Es belegt die gute Trennung der einzelnen Substanzen und die Eignung von Diazepam als innerem Standard. Die Fig. 2–4 zeigen die Trennungen der wichtigsten in *Digitalis purpurea*, *Digitalis heywoodii* und in einer Hybride *Digitalis heywoodii* × *Digitalis lanata* vorkommenden Glykoside.

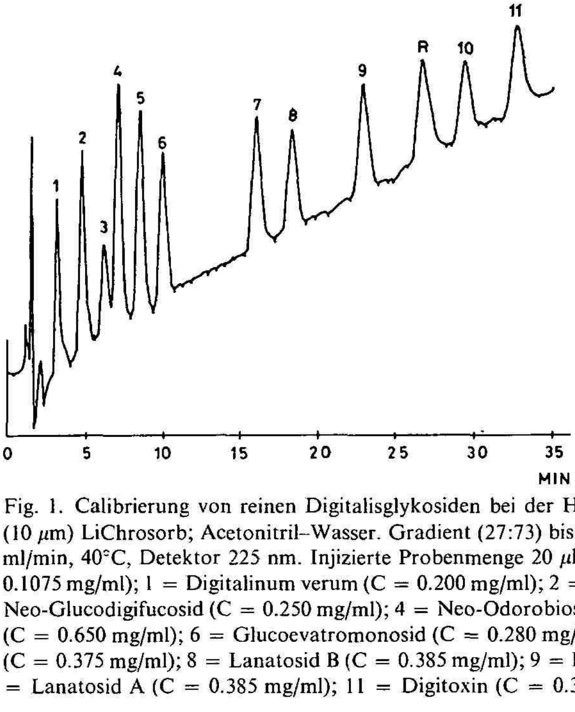


Fig. 1. Calibrierung von reinen Digitalisglykosiden bei der HPLC-Analyse. Bedingungen: RP-18-Säule (10 μm) LiChrosorb; Acetonitril–Wasser. Gradient (27:73) bis (39:61) innerhalb 35 min, linear; Fluss 1.5 ml/min, 40°C, Detektor 225 nm. Injizierte Probenmenge 20 μl. R = Diazepam (Innerer Standard, C = 0.1075 mg/ml); 1 = Digitalinum verum (C = 0.200 mg/ml); 2 = Glucoverodoxin (C = 0.425 mg/ml); 3 = Neo-Glucodigifucosid (C = 0.250 mg/ml); 4 = Neo-Odorobiosid G (C = 0.360 mg/ml); 5 = Lanatosid C (C = 0.650 mg/ml); 6 = Glucoevatromonosid (C = 0.280 mg/ml), 7 = Glucodigitoxigeninbisdigitoxosid (C = 0.375 mg/ml); 8 = Lanatosid B (C = 0.385 mg/ml); 9 = Purpureaglykosid A (C = 0.415 mg/ml); 10 = Lanatosid A (C = 0.385 mg/ml); 11 = Digitoxin (C = 0.325 mg/ml).

Um sich über die Reproduzierbarkeit der Methode ein Bild zu machen, haben wir aus sieben Analysen einer homogenen Probe von *Digitalis heywoodii*-Blättern, von den einzelnen Werten ausgehend die Standardabweichungen berechnet und in Tabelle I zusammengestellt. Wie man daraus ersieht, ist die Streuung der Werte relativ gering und eine Störung durch noch vorhandene Ballaststoffe kaum zu befürchten.

Vergleich mit der Methode von Kaiser[2]

Wir haben die von uns vorgeschlagene quantitative HPLC-Analyse mit der quantitativen Analyse von Digitalisblattextrakten nach Kaiser[2] (Kombination Säulen–Papierchromatographie, photometrische Bestimmung über die Farbreaktion nach Baljet[3,4]) verglichen. Wie Tabelle II ausweist, sind die beobachteten Differenzen gering. Die HPLC-Analyse bietet gegenüber dem Verfahren von Kaiser zwei wesentliche Vorteile: der Bedarf an Probenmaterial kann von 50 g auf 1.5 g reduziert werden und die Analysendauer lässt sich sehr stark, von *ca.* 35 h auf *ca.* 2–3 h verkürzen.

TABELLE I

EINZELWERTE, MITTELWERT UND STANDARDABWEICHUNG BEI DER QUANTITATIVEN HPLC-ANALYSE VON *DIGITALIS HEYWOODII*-BLÄTTERN.

Alle Angaben in mg/100 g trockener Blattdroge.

	Einzelwerte							*Mittelwert*	*Standardabweichung*[5]
	1	*2*	*3*	*4*	*5*	*6*	*7*		
Digitalinum verum	42	39	41	38	42	37	36	39	± 2.44
Glucoverodoxin	41	42	46	44	44	46	45	44	± 1.91
Glucodigifucosid	22	21	22	23	22	21	23	22	± 0.81
Odorobiosid G	22	24	22	25	23	24	21	23	± 1.41
Glucoevatromonosid	194	198	204	205	200	196	196	199	± 4.20
Lanatosid B	138	139	140	136	135	136	135	137	± 2.00
Purpureaglykosid A	594	597	606	602	589	614	584	598	± 10.24
Lanatosid A	408	414	411	407	405	416	416	411	± 4.47
Digitoxin	67	69	69	72	70	65	71	69	± 2.38

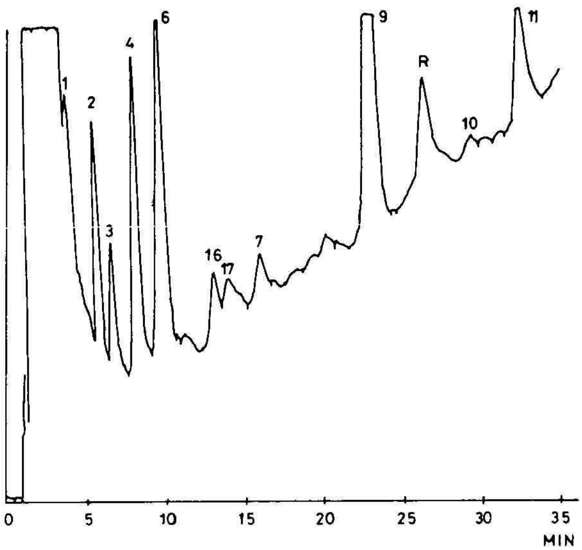


Fig. 2. HPLC-Trennung eines Blattextraktes aus *Digitalis purpurea* L. Bedingungen: s. Fig. 1. R = Innerer Standard Diazepam (C = 0.027 mg/ml); 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Neo-Glucodigifucosid; 4 = Neo-Odorobiosid G; 6 = Glucoevatromonosid; 7 = Glucodigitoxigeninbisdigitoxosid; 9 = Purpureaglykosid A; 10 = Lanatosid A; 11 = Digitoxin; 16 = Purpureaglykosid B; 17 = nicht identifiziert.

DANKSAGUNG

Wir danken der Deutschen Forschungsgemeinschaft (Bonn–Bad Godesberg) für die Bereitstellung des HPLC-Gerätes und für finanzielle Unterstützung. Herrn Dr. F. Kaiser, Boehringer-Mannheim danken wir für die Überlassung von Glucoeva-

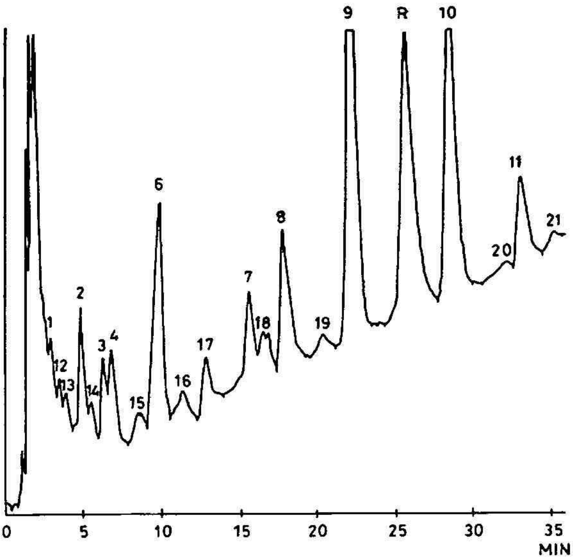


Fig. 3. HPLC-Trennung von *Digitalis heywoodii* P. et M. Silva. Bedingungen: s. Fig. 1. R = Innerer Standard Diazepam (C = 0.0625 mg/ml); 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Neo-Glucodigifucosid; 4 = Neo-Odorobiosid G; 6 = Glucoevatromonosid; 7 = Glucodigitoxigeninbisdigitoxosid; 8 = Lanatosid B; 9 = Purpureaglykosid A; 10 = Lanatosid A; 11 = Digitoxin; 12, 13, 14 = nicht identifiziert; 15 = Digoxin; 16 = Desacetyllanatosid B; 17 = *β*-Acetyldigoxin; 18 = *α*-Acetyldigoxin; 19 = Acetylgitoxin; 20, 21 = nicht identifiziert.

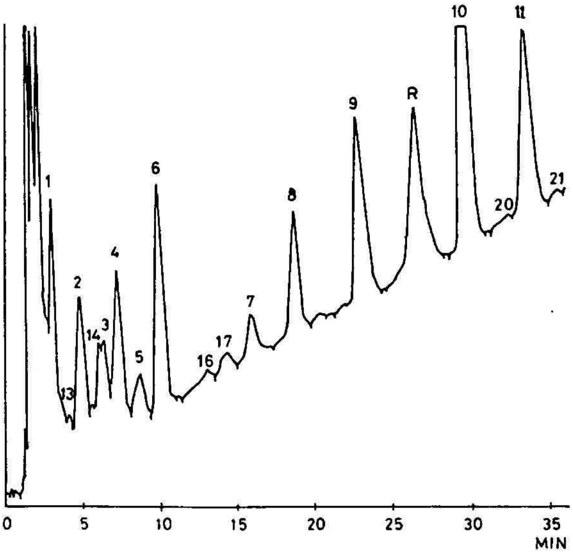


Fig. 4. HPLC-Trennung von *Digitalis heywoodii* P. et M. Silva × *Digitalis lanata* EHRH. Bedingungen: s. Fig. 1. R = Innerer Standard Diazepam (C = 0.0535 mg/ml); 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Neo-Glucodigifucosid; 4 = Neo-Odorobiosid G; 5 = Lanatosid C; 6 = Glucoevatromonosid; 7 = Glucodigitoxigeninbisdigitoxosid; 8 = Lanatosid B; 9 = Purpureaglykosid A; 10 = Lanatosid A; 11 = Digitoxin; 13, 14 = nicht identifiziert; 16 = Desacetyllanatosid B; 17 = Acetyldigoxin; 20, 21 = nicht identifiziert.

TABELLE II

VERGLEICH DER NACH KAISER[2] UND MITTELS QUANTITATIVER HPLC ERHALTENEN WERTE BEI DER GLYKOSIDBESTIMMUNG IN BLÄTTERN VERSCHIEDENER DIGITALIS-ARTEN UND HYBRIDE; ES SIND NUR MENGENMÄSSIG BEDEUTENDE GLYKOSIDE ANGEGEBEN

Mittelwerte aus jeweils 4 bis 7 HPLC-Analysen. Alle Angaben in mg/100 g trockener Blattdroge.

	D. purpurea		*D. lutea*		*D. purpurea* × *D. lutea*		*D. lutea* × *D. purpurea*		*D. heywoodii*		*D. heywoodii* × *D. lanata*	
	Kaiser	*HPLC*	*Kaiser*	*HPLC*	*Kaiser*	*HPLC*	*Kaiser*	*HPLC*	*Kaiser*	*HPLC*	*Kaiser*	*HPLC*
Digitalinum verum	47	43	15	11	58	47	45	47	46	39	18	21
Glucoverodoxin	23	19	3	6	17	20	14	12	42	44	68	73
Glucodigifucosid (einsch. Neo-Verbindung)	12	9	Spuren	–	Spuren	3	Spuren	Spuren	22	22	21	19
Odorobiosid G (einschl. Neo-Verbindung)	43	37	8	6	78	72	70	79	22	23	71	69
Lanatosid C	–	–	Spuren	3	Spuren	–	Spuren	–	–	–	10	11
Glucoevatromonosid	55	49	14	15	73	70	89	77	222	199	116	101
Lanatosid B	–	–	8	10	12	10	16	20	133	137	36	34
Purpureaglykosid A	75	79	–	–	11	9	67	60	680	598	98	89
Lanatosid A	–	2	22	24	38	43	33	35	426	411	240	256
Digitoxin	18	21	20	18	17	22	13	13	72	69	89	92

tromonosid, Glucogitorosid, Glucolanadoxin, Glucoverodoxin und Glucogitofucosid, Herrn Dr. A. Angliker, Sandoz AG, Basel, für die Lanatoside A, B, C und für Digitalinum verum.

NOTIZ

Nach Abschluss dieser Arbeit erhielten wir Kenntnis von der Publikation von Jurenitsch *et al.*[6] über die HPLC-Analyse von Convallaria-Glykosiden. Die Autoren kommen methodisch (Probenvorbereitung, mobile Phase, Detektion) zu sehr ähnlichen Resultaten; ihre Ergebnisse bestätigen eindrucksvoll die Überlegenheit der HPLC in der Herzglykosidanalytik gegenüber den bisherigen Verfahren.

LITERATUR

1 M. Wichtl, M. Mangkudidjojo und W. Wichtl-Bleier, *J. Chromatogr.*, 234 (1982) 503.
2 F. Kaiser, *Arch. Pharm. (Weinheim)*, 299 (1966) 263.
3 H. Baljet, *Pharm. Weekbl.*, 55 (1918) 457.
4 L. Fuchs, M. Wichtl und H. Jachs, *Arch. Pharm. (Weinheim)*, 291 (1958) 193; 292 (1959) 15.
5 E. Kreyszig, *Statistische Methoden und ihre Anwendungen*, Verlag Vandenhoek und Ruprecht, 3. Aufl. Göttingen 1968.
6 J. Jurenitsch, B. Kopp, E. Bamberg-Kubelka und W. Kubelka, *J. Chromatogr.*, 240 (1982) 235.

Journal of Chromatography, 247 (1982) 366–368
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,121

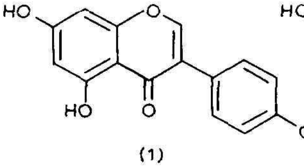
Note

Quantitative analysis of the isoflavone phyto-oestrogens genistein, formononetin and biochanin A, in subterranean clover leaves by high-performance liquid chromatography

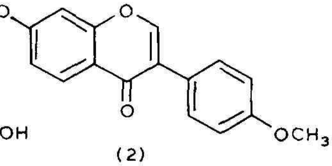
JOSEPH J. PATRONI*,*
Department of Organic Chemistry, University of Western Australia, Nedlands, W.A. 6009 (Australia)
and
W. J. COLLINS** and W. R. STERN
Department of Agronomy, Institute of Agriculture, University of Western Australia, Nedlands, W.A. 6009 (Australia)

(Received June 17th, 1982)

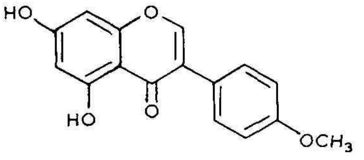
Oestrogenic activity in subterranean clover (*Trifolium subterraneum* L.) has been recognised for many years as a major cause in infertility problems in sheep[1]. Success in alleviating these problems has been due largely to the development, through breeding and selection, of lines of subterranean clover with low levels of oestrogenic activity. An essential part of this programme is the monitoring of levels of isoflavones on a large number of lines. Leaves of clover are assayed for the isoflavone phyto-oestrogens genistein (1), formononetin (2) and biochanin A (3). Originally, all three compounds were thought to be implicated in the infertility problem, but there is now strong evidence to suggest that formononetin alone, is the main factor involved[2].



(1)



(2)



(3)

---

* Present address: Sugar Research Institute, Mackay, Queensland 4740, Australia.

** Present address: Western Australian Department of Agriculture, South Perth, W.A. 6151, Australia.

The analytical method used in the isoflavone laboratory is based on the visual estimation of developed thin-layer chromatographic plates[3]. The method is semi-quantitative and was developed from the chromatographic method of Beck[4] and an extraction technique modified from that of Curnow[5].

It is some years since an accurate analysis of the levels of isoflavones in subterranean clover was undertaken. For this purpose we used high-performance liquid chromatographic (HPLC) procedures. The high ultraviolet absorption (λ_{max}263–264 nm), coupled with the moderately polar nature of these isoflavones were considered ideal characteristics for the application of UV-monitored C_{18} reversed-phase chromatography with a polar eluent such as methanol–water.

This paper describes the successful resolution and quantitation of the isoflavones 1, 2 and 3 found in fresh clover leaves.

EXPERIMENTAL

Materials and apparatus

All solvents were analytical-reagent grade.

HPLC was with a Waters Assoc. (Milford, MA, U.S.A.) M-6000A pump fitted with a μBondapak™ C_{18} (30 cm × 3.9 mm I.D.) pre-packed column and a U6K loop injector. Detection was by elution monitoring with a Waters 440 absorbance detector (254 nm, 0.5 a.u.f.s.). Output was measured on a Hewlett-Packard 3380A integrator using the external-standard area quantitation method. Manual injection was with a Hamilton 800 (25 μl) syringe with the chromatographic system at ambient temperature.

Two standard solutions consisting of a mixture of isoflavones 1, 2 and 3 in methanol (250 μg/ml and 50 μg/ml) were stored at 2°C for a period of 3 months without decomposition.

Procedure

Twelve circular clips of undamaged subterranean clover leaves were ground with a glass rod and acid-washed sand (0.1 g). Ethanol (1 ml) was then added and the mixture allowed to stand in a water bath (60°C, 10 min). The extract was decanted and the extraction procedure repeated on the residue to give a combined total extract (2.0 ml) as described previously[3]. Part of the solution (1.0 ml) was then evaporated to 0.5 ml and applied to Sep-Pak™ C_{18} cartridges, pre-washed with methanol, and finally eluted with methanol (4 × 1 ml). This eluent was concentrated to dryness (water bath at 60°C) and made up to a final volume with methanol (2.0 ml).

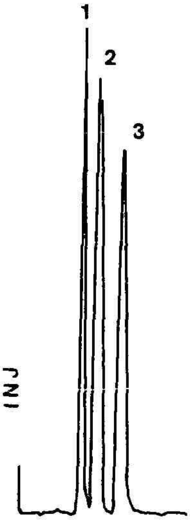
Reversed-phase HPLC [methanol–water (27:73) at a flow-rate of 2.5 ml/min, 2500 p.s.i.] of the prepared extract (20-μl injections), gave symmetrical peaks with baseline resolution of the isoflavones 1, 2 and 3. Detector response area was shown to be linear over the relevant range (0.5–5.0 μg) for each of the three standards. Quantitation was therefore determined by calibration of the on-line integrator by an external-standard mixture prior to analytical runs. Reproducibility of quantitation of a standard mixture of 1, 2 and 3 subjected to this analytical procedure was within 5% at the 1-μg level.

RESULTS AND DISCUSSION

The system described allowed analysis of some 1000 clover-leaf extracts with negligible loss of column performance.

Typical chromatograms obtained from standard solution (Fig. 1) and from extract (Fig. 2) gave symmetrical, well-resolved peaks. Over a period of 3 months retention times of compounds 1, 2 and 3 varied only slightly and within the ranges 1.52–1.59, 1.82–1.96, 2.26–2.57 min, respectively.

The levels of isoflavones determined for a range of subterranean clover cultivars will be reported in a separate publication.



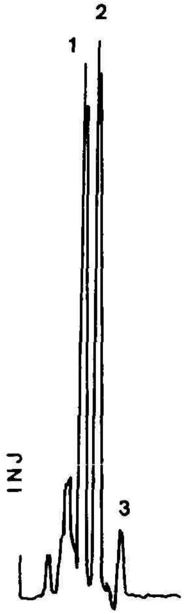


Fig. 1. HPLC separation of a standard mixture of genistein (1) (996 ng), formononetin (2) (1008 ng), and biochanin A (3) (1024 ng).

Fig. 2. HPLC separation of a typical clover leaf extract. Peaks: 1 = genistein (1040 ng); 2 = formononetin (1110 ng); 3 = biochanin A (180 ng). Chromatographic conditions are described in the Experimental section.

ACKNOWLEDGEMENTS

We thank Professor P. R. Jefferies for advice and access to the HPLC equipment.

The Wool Research Trust Fund provided financial support.

REFERENCES

1 G. R. Moule, A. W. H. Braden and D. R. Lamond, *Anim. Breed. Abst.*, 31 (1963) 139.
2 A. W. H. Braden and D. A. Shutt, in M. J. T. Norman (Editor), *Proceedings of the XIth International Grassland Congress, 1970, Surfers Paradise, Australia*, University of Queensland Press, 1970, p. 770.
3 C. M. Francis and A. J. Millington, *Aust. J. Agr. Res.*, 16 (1965) 557.
4 A. B. Beck, *Aust. J. Agr. Res.*, 15 (1964) 223.
5 D. H. Curnow, *Biochem. J.*, 58 (1954) 283.

Journal of Chromatography, 247 (1982) 369–373
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

CHROM. 15,130

Note

Liquid chromatography of some polyurethane polyols

G. BRUCE GUISE* and GEOFFREY C. SMITH
CSIRO Division of Textile Industry, Belmont, Victoria 3216 (Australia)

(Received June 21st, 1982)

Polyurethanes are a diverse family of polymers but all are derived from two types of intermediates, diisocyanates and polyols. A number of chromatographic methods have been described for the analysis of the commonly used diisocyanates but little has been published on polyols even though they represent about 75% (w/w) of most polyurethanes. Most polyurethanes are based on two diisocyanates, 2,4-tolylene diisocyanate (TDI) and diphenylmethane diisocyanate (MDI), but a wide range of polyols is used.

The most common type of polyols are poly(propylene oxide) (PPO) and these are manufactured by anionic polymerisation of propylene oxide and polyhydric alcohols (*e.g.* glycerol, propan-1,2-diol, trimethylol-propane). Two side reactions occur in their manufacture: (1) with water (present as impurity) to form PPO diols and (2) chain transfer to monomer which forms allyl alcohol, and this results in the production of PPO mono-ol. Thus, commercial PPO triols contain diol and mono-ol impurities, and the average functionality is below 3.

PPO triols showed a single narrow peak on gel permeation chromatography[1] (GPC), but with thin-layer chromatography (TLC) on silica gel, separation of the mono-ol and diol impurities has been observed[2–4] by Russian workers. This note reports an adaption of the TLC method[2–4] to high-performance liquid chromatography (HPLC). As well as PPO polyols, some other types of polyol were examined. The method was used to follow the reaction of a PPO triol with isocyanates.

EXPERIMENTAL

Polyols were obtained from Bayer (Leverkusen, G.F.R.) and Union Carbide (Melbourne, Australia); the PPO triol (molecular weight, MW = 3000) was Desmophen 3400 (Bayer). The TDI was Desmodur T100 (Bayer) and all other chemicals were analytical grade.

Chromatography was carried out using a 150 × 4.6 I.D. mm column packed with 5-μm LiChrosorb SI 60 (Merck). The pump was a DuPont Model 870, the injection system was supplied by Valco, and the detector was a Shodex SE-11 (Showa Denko KK, Tokyo, Japan) refractive index detector.

After examining a number of solvents and mixtures, we found that ethyl acetate containing a small amount of a polar solvent (methanol or propan-2-ol) gave the best resolution. Russian workers[2–4] used ethyl acetate–water–butan-2-one for their TLC work, but this was too polar for HPLC. Ethyl acetate containing 1%

0021-9673/82/0000-0000/$02.75

propan-2-ol was used in the experiments described below. Samples were injected (10-μl loop) in ethyl acetate and the flow-rate was either 2 ml/min or 0.5 ml/min.

Reactions of the triol with the isocyanates were carried out in the absence of solvent under an atmosphere of nitrogen (see legends to figures for further details).

RESULTS AND DISCUSSION

In the Russian study[2–4] of the TLC of PPO polyols, the extent of adsorption was found to be determined by the number of hydroxyl groups, and for a given

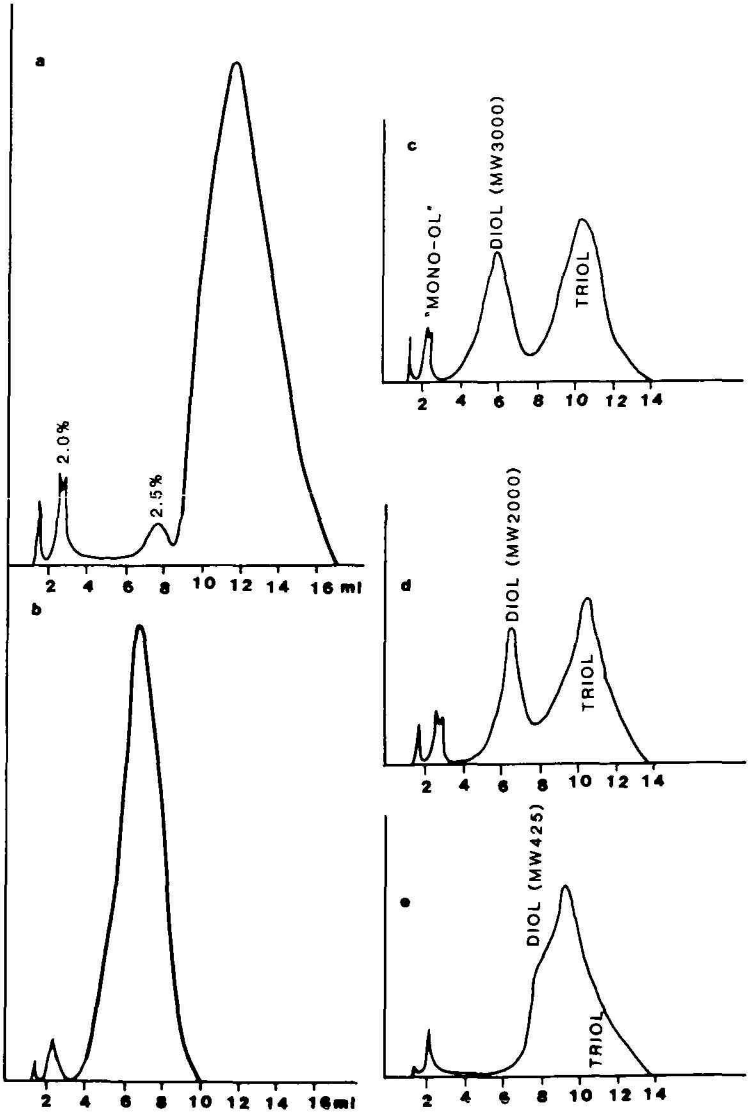


Fig. 1. HPLC of PPO triol (MW 3000) and diols. (a) Triol alone; (b) diol (MW 2000) alone; (c) triol + diol (MW 3000) (3:1); (d) triol + diol (MW 2000) (3:1); (e) triol + diol (MW 425) (3:1).

molecular weight the R_F values were mono-ol > diol > triol, and for a given functionality the R_F value increased with increasing molecular weight. In the present HPLC study, similar trends were assumed, and peaks (Fig. 1a) eluted in front of the mean peak of a MW 3000 triol were assigned to diol and mono-ol on this basis. The mono-ol peak was split, and ahead of it was another peak which appeared to be due to a volatile impurity as it was lost by heating at 80°C for several hours. The resolution of a MW 3000 triol from diols of various molecular weights is shown in Fig. 1c–e. The HPLC method appears to be capable of detecting as little as either 1% diol or mono-ol impurities in PPO triols (that is, assuming equal detector responses of the components). The amount of diol detected in the PPO triol (MW 3000) used in this work was much lower than found[2–4] by TLC in a similar polymer; this difference was attributed to the manufacturing method rather than the analytical method.

A common modification of PPO polyols is to terminate the chains with ethylene oxide units. This replaces the terminal secondary hydroxyls with primary hydroxyls which react faster with isocyanates. On HPLC, such polyols were found to adsorb much more strongly than pure propylene oxide polyols, but they could be eluted by the addition of more isopropanol to the solvent. However, the resultion between triol, diol and mono-ol decreased as the proportion of ethylene oxide units increased, and the amounts of lower functionality impurities could no longer be estimated. The main value of HPLC with ethylene oxide-tipped PPO polyols is to give an indication of the presence of ethylene oxide units.

Some polyester polyols were examined by HPLC. These were similar in polarity to the PPO polyols, and an eluent containing less propan-2-ol was used. A poly(ethylene adipate), MW 2000, showed mainly as a sharp peak; the splitting of the tail of this peak was considered to be due to the separation of the more polar low-molecular-weight oligomers. Some polycaprolactone polyols (from Union Carbide) showed quite extensive splitting and this was assumed to be separation of oligomers; a similar separation of oligomers has recently been reported with hydroxy functional poly(methyl methacrylates)[5].

The HPLC method has been used to follow the reaction of a PPO triol (MW 3000) with isocyanates. In Fig. 2, chromatograms of the reaction product of this triol with different amounts of *n*-butylisocyanate are shown. The peaks in order of elution were assigned to the products of the reaction of the triol with 3, 2 and 1 mol of *n*-butylisocyanate, respectively and were well separated. The GPC method described previously[1] does not resolve these components. The HPLC method could no doubt be used to follow the kinetics of these reactions.

In many applications, polyols are used directly to form polyurethanes, but in others they are first reacted with diisocyanates to form isocyanate terminated prepolymers which are subsequently converted into polyurethanes. The formation of such a prepolymer from PPO triol (MW 3000) and TDI was followed by HPLC (Fig. 3). Control experiments showed that there was negligible reaction of the isocyanates with the propan-2-ol solvent during a chromatogram. After 2 h at 80°C the isocyanate content (determined by reaction with excess amine and titration with acid) of the triol–TDI reaction mixture was constant[1], and this is normally taken to indicate complete reaction. However, HPLC showed that some of the intermediate reaction products were still present. In this reaction, higher-molecular-weight products are formed by chain extension (*e.g.* from 2 mol of triol and 5 mol of diisocyanate), and

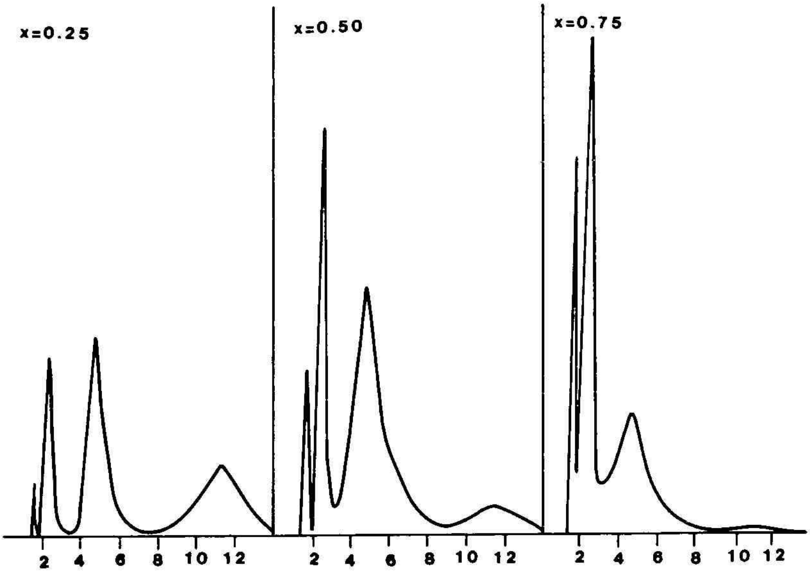


Fig. 2. HPLC of reaction product of PPO triol (MW 3000) and x mol n-butylisocyanate (+ trace of dibutyltin dilaurate catalyst) at 20°C for 16 h.

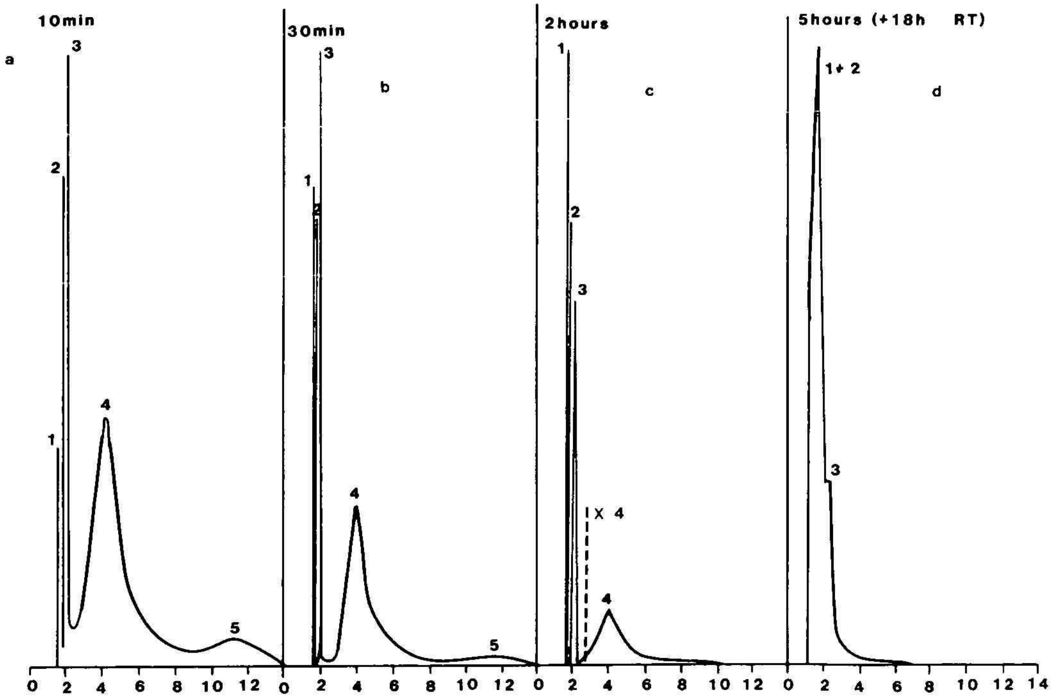


Fig. 3. Reaction product of PPO triol (MW 3000) with TDI (3 mol) at 80°C (no catalyst). (a) After 10 min (attenuation 4); (b) 30 min (attenuation 4); (c) 2 h (attenuations 8 and 2); (d) 5 h (attenuation 8). Peaks: 1 = excluded material; 2 = trisubstituted triisocyanate + TDI; 3 = disubstituted (mono-ol); 4 = monosubstituted (diol); 5 = triol. Horizontal axis denotes time in min, RT = room temperature (20°C).

these products are well resolved by GPC, but not by HPLC. The peak (Fig. 3c) corresponding to the fully reacted product was split on HPLC; this could be due to unreacted TDI, but on the silica used (pore size 6 nm) there is also the possibility of size exclusion of the high-molecular-weight chain extended products.

HPLC thus can be used for the characterization and analysis of polyurethane polyols and to follow their reaction with isocyanates. Lower functionality impurities could be estimated in PPO triols. This HPLC method appears to be a useful complement to GPC studies for polyurethane polyols and the isocyanate terminated prepolymers derived from them.

REFERENCES

1 G. B. Guise and G. C. Smith, *J. Chromatogr.*, 214 (1981) 69.

2 B. G. Belenky, M. D. Valchikhina, I. A. Vakhtina, E. S. Gankina and O. G. Tarakanov, *J. Chromatogr.*, 129 (1976) 115.

3 I. A. Vakhtina, Y. A. Petrakova, V. Pentsel, O. G. Tarakanov, *Polym. Sci. USSR (Engl. Transl.)*, 22 (1980) 1833.

4 K. Knopp, G. A. Gladkovskii, Y. A. Petrakova, U. Pentsel, I. A. Vakhtina and V. S. Lebeder, *Polym. Sci. USSR (Engl. Transl.)*, 22 (1980) 1958.

5 G. D. Andrews and A. Vatyars, *Macromolecules*, 14 (1981) 1603.

Journal of Chromatography, 247 (1982) 374
Elsevier Scientific Publishing Company, Amsterdam -- Printed in The Netherlands

CHROM. 15,065

Letter to the Editor

Conversion of linear into logarithmic retention indices

Sir,

The use of the linear retention index (J) in isothermal gas chromatography was initially proposed in 1968[1]. The plot for the conversion of linear into logarithmic (I) retention indices was also given in the above paper. The equation

$$\frac{R^{\delta I/100} - 1}{R - 1} = \delta J \tag{1}$$

where $\delta I = I - 100\,z$ and $\delta J = J - z$, was given in 1971[2]; R is the relation of the adjusted retention times of neighbouring *n*-alkanes and z is the carbon number of the first *n*-alkane standard. In a book[3], a detailed table was given constructed according to the above equation. A review[4] included problems with the use of the linear retention index in gas chromatography.

Recently Mitra[5] rewrote eqn. 1 incorrectly, replacing δJ by J, and asserted that it was in that form that the above equation was given in ref. 2. Then Mitra made the corresponding "correction" and obtained finally an equation identical with eqn. 1. It is evident that if Mitra had read the earlier publications[2–4] more attentively he would not have felt the necessity to publish his note[5].

Kuibyshev State University,
Chemical Department,
Kuibyshev-86 (U.S.S.R.)

M. S. VIGDERGAUZ

1 M. S. Vigdergauz, in H. G. Struppe (Editor), *Gas-Chromatographie 1968 —Vorträge des VI. Symposiums über Gas Chromatographie, Berlin, Mai 1968*, Akademie-Verlag, Berlin, 1968, p. 625.
2 M. S. Vigdergauz and A. A. Martynov, *Chromatographia*, 4 (1971) 463.
3 M. S. Vigdergauz, *Raschety v gazovoj khromatografii (Calculations in Gas Chromatography)*, Khimiya, Moscow, 1978 (in Russian).
4 M. S. Vigdergauz, *Zh. Anal. Khim.*, 31 (1976) 2222.
5 G. D. Mitra, *J. Chromatogr.*, 211 (1981) 239.

(Received February 18th, 1982)

Journal of Chromatography, 247 (1982) 375
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,066

Letter to the Editor

Conversion of linear into logarithmic retention indices

Sir,

The equation for conversion of linear (*J*) into logarithmic (*I*) retention indices given by Vigdergauz in his Letter to the Editor is correct and identical with the modified relationship published by myself[1]. The comment of Vigdergauz that "Mitra rewrote eqn. 1 incorrectly, replacing δJ by *J*" is inaccurate. The fact is that Soják and Vigdergauz[2] have quoted the incorrect equation using *J* instead of δJ themselves, from the earlier work of Vigdergauz and Martynov[3] (see the first line on page 160 of ref. 2), stating, "The analytical relationship between the quantities *J* and *I* has the following form[5]". I felt that Vigdergauz might have given the incorrect equation (eqn. 3), which needs modification, and thus my paper on this point was published[1]. In fact, I did not claim the equation to be my own rather than a modification. Further, if the said equation was misprinted in ref. 2, the authors could have published an addendum or erratum, rather than criticizing me and others. It was necessary to go through the validity of the equation when it has been published in a reputable international journal[2], particularly when quoted by the original author.

In scientific fields such mistakes may happen unknowingly, but should be accepted with good grace when they are pointed out.

Gas Chromatography Laboratory, Fertilizer (P&D) India Ltd., Sindri, Dhanbad, Bihar (India) G. D. MITRA

1 G. D. Mitra, *J. Chromatogr.*, 211 (1981) 239.
2 L. Soják and M. S. Vigdergauz, *J. Chromatogr.*, 148 (1978) 159.
3 M. S. Vigdergauz and A. A. Martynov, *Chromatographia*, 4 (1971) 463.

(Received March 15th, 1982)

Author Index

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	241/2 242/1 242/2	243/1 243/2 244/1 244/2	245/1 245/2 245/3	246/1 246/2 247/1	247/2 248/1 248/2 248/3	249/1 249/2 250	
Chromatographic Reviews		251/1		251/2								251/3
Biomedical Applications	227/1	227/2	228	229/1	229/2	230/1	230/2	231/1	231/2	232/1	232/2	233

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 244, No. 2, pp. 401–404. A free reprint can be obtained by application to the publisher.)

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), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of 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. (Short communications and Notes 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 lay-out 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", "submitted for publication", "in preparation" or "personal communication".

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, Short communications and Notes 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.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.