ISSN-0021-9673

VOL. 176 NO. 1 AUGUST 1, 1979

RNAL OF

HROMATOGRAPHY

RNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

EDITOR, Michael Lederer (Rome)

ASSOCIATE EDITOR, K. Macek (Prague)

EDITORIAL BOARD
W. A. Aue (Halifax)
V. G. Berezkin (Moscow)
V. Betina (Bratislava)
A. Bevenue (Honolulu, Hawaii)
P. Boulanger (Lille)
A. A. Boulton (Saskatoon)
G. P. Cartoni (Rome)
G. Duyckaerts (Liège)
L. Fishbein (Jefferson, Ark.)
A. Frigerio (Milan)
C. W. Gehrke (Columbia, Mo.)
E. Gil-Av (Rehovot)
G. Guiochon (Palaiseau)
I. M. Hais (Hradec Králové)
E. Heftmann (Berkeley, Calif.)
S. Hjertén (Uppsala)
E. C. Horning (Houston, Texas)
Cs. Horváth (New Haven, Conn.)
J. F. K. Huber (Vienna)
A. T. James (Sharnbrook)
J. Janák (Brno)
E.sz. Kováts (Lausanne)
K. A. Kraus (Oak Ridge, Tenn.)
E. Lederer (Gif-sur-Yvette)
A. Liberti (Rome)
H. M. McNair (Blacksburg, Va.)
Y. Marcus (Jerusalem)
G. B. Marini-Bettòlo (Rome)
Č. Michalec (Prague)
R. Neher (Basel)
G. Nickless (Bristol)
J. Novák (Brno)
N. A. Parris (Wilmington, Del.)
P. G. Righetti (Milan)
O. Samuelson (Göteborg)
G.-M. Schwab (Munich)
G. Semenza (Zürich)
L. R. Snyder (Tarrytown, N.Y.)
A. Zlatkis (Houston, Texas)

EDITORS, BIBLIOGRAPHY SECTION
K. Macek (Prague), J. Janák (Brno), Z. Deyl (Prague)

EDITOR, BOOK REVIEW SECTION
R. Amos (Abingdon)

EDITOR, NEWS SECTION
J. F. K. Huber (Vienna)

COORD. EDITOR, DATA SECTION
J. Gasparič (Hradec Králové)

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1979

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1978	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	166/1 166/2 167	168/1 168/2	169 170/1	170/2	171 172	173/1 173/2	174/1	174/2 175/1 175/2	176/1 176/2	176/3 177/1 177/2	The publication schedule for the volumes 178–180 will be published later.		
Chromatographic Reviews				165/1			165/2				165/3		
Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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, if possible 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.
Reviews 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.

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, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 19 volumes in 1979. The subscription price for 1979 (Vols. 162–180) is Dfl. 2356.00 plus Dfl. 285.00 (postage) (total *ca.* US$ 1288.00). The subscription price for the *Biomedical Applications* section only (Vols. 162–164) is Dfl. 384.00 plus Dfl. 45.00 (postage) (total *ca.* US$ 209.00). Journals are automatically sent by air mail 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 161) are available at Dfl. 140.00 (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/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

© ELSEVIER SCIENTIFIC PUBLISHING COMPANY — 1979

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the publisher, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Submission of an article for publication implies the transfer of the copyright from the author to the publisher and is also understood to imply that the article is not being considered for publication elsewhere.

Submission to this journal of a paper entails the author's irrevocable and exclusive authorization of the publisher to collect any sums of 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.

Printed in The Netherlands

CONTENTS

Parameter study of a hydrogen atmosphere flame ionization detector
by J. E. Roberts and H. H. Hill, Jr. (Pullman, Wash., U.S.A.) (Received February 2nd, 1979) 1

Regenerable affinity chromatography support
by V. K. Joshi and K. M. Shahani (Lincoln, Nebr., U.S.A.), A Kilara (University Park, Pa., U.S.A.) and F. W. Wagner (Lincoln, Nebr., U.S.A.) (Received March 23rd, 1979) 11

Electron capture gas chromatography with splitless injection on isothermally operated wide-bore glass capillary columns
by H. Brötell (Södertälje, Sweden), N.-O. Ahnfelt (Uppsala, Sweden) and H. Ehrsson and S. Eksborg (Stockholm, Sweden) (Received March 22nd, 1979). 19

Use of specific retention volumes in the evaluation of various types of columns for use in the trace determination of ethylene glycol by gas chromatography
by M. E. Kashtock (Washington, D.C., U.S.A.) (Received February 13th, 1979) 25

Selection of a gas chromatographic material for use in explosives vapor preconcentration
by F. J. Conrad, T. A. Burrows and W. D. Williams (Albuquerque, N. M., U.S.A.) (Received March 26th, 1979) 37

Experimental study of selectivity and column efficiency in clathrate chromatography using Werner complexes as clathrate host components. I. Relationship between selectivity of $Ni(NCS)_2(4\text{-methylpyridine})_4 \cdot$ guest clathrate sorbents and composition of the mobile phase
by J. Lipkowski, M. Pawłowska and D. Sybilska (Warsaw, Poland) (Received March 9th, 1979). 43

N-Chloromethylphthalimides as derivatization reagents for high-performance liquid chromatography
by W. Lindner (Graz, Austria) and W. Santi (Basle, Switzerland) (Received February 26th, 1979) 55

Liquid column chromatography of nucleoside monophosphates on DEAE-Spheron 1000
by V. Svoboda and I. Kleinmann (Prague, Czechoslovakia) (Received March 26th, 1979) 65

Quantitation of polymethoxylated flavones in orange juice by high-performance liquid chromatography
by R. L. Rouseff and S. V. Ting (Lake Alfred, Fla., U.S.A.) (Received March 2nd, 1979) 75

Synthesis of highly diversified carrier ampholytes. Evaluation of the resolving power of isoelectric focusing in the Pi system (alpha-1-antitrypsin genetic polymorphism)
by R. Charlionet, J. P. Martin, R. Sesboüé, P. J. Madec and F. Lefebvre (Bois-Guillaume, France) (Received March 19th, 1979) 89

Determination of spectinomycin by high-performance liquid chromatography with fluorometric detection
by H. N. Myers and J. V. Rindler (Kalamazoo, Mich., U.S.A.) (Received March 27th, 1979). 103

Use of reversed-phase high-performance liquid chromatographic analysis for the determination of provitamin A carotenes in tomatoes
by M. Zakaria, K. Simpson and P. R. Brown (Kingston, R. I., U.S.A.) and A. Krstulovic (Purchase, N.Y., U.S.A.) (Received March 28th, 1979). 109

Notes

Gas chromatographic determination of low-molecular-weight carbonyl compounds in aqueous solution as their pentafluorophenylhydrazones
by K. Kobayashi, M. Tanaka, S. Kawai and T. Ohno (Gifu, Japan) (Received February 23rd, 1979) 118

(Continued overleaf)

18.MRT.1982

Contents (continued)

Gas chromatographic determination of preservatives in rennet
by A. Geahchan, M. Pierson and P. Chambon (Lyon, France) (Received March 1st, 1979) 123

Gas chromatographic analysis of 1-monoacylglycerols as cyclic carbonate derivatives
by J. Oehlenschläger and G. Gercken (Hamburg, G.F.R.) (Received March 22nd, 1979) 126

Gas chromatographic determination of ajmaline in the bark of the root of *Rauvolfia vomitoria*
by G. P. Forni (Milan, Italy) (Received February 26th, 1979) 129

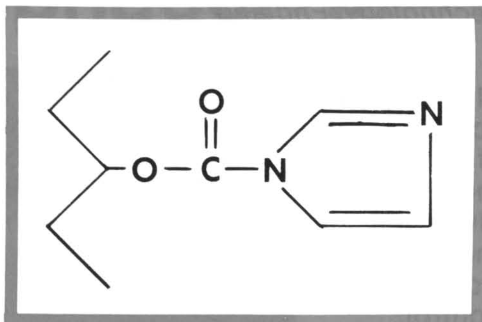
Sensitive gas chromatographic method for the determination in blood and urine of SL 75212 [4-(2-cyclopropylmethoxyethyl)-1-phenoxy-3-isopropylaminopropan-2-ol], a new β_1 adrenoceptor blocking agent
by G. Bianchetti, J. Ganansia and P. L. Morselli (Paris, France) (Received March 1st, 1979) 134

Analyse par chromatographie liquide haute pression de la décaméthrine
par C. Meinard, J. C. Suglia et P. Bruneau (Marseille, France) (Reçu le 27 février 1979) . 140

Identification and separation of closely related sulfa drugs by thin-layer chromatography on cadmium acetate-impregnated silica gel plates
by S. P. Srivastava, V. K. Dua, R. N. Mehrotra and R. C. Saxena (Roorkee, India) (Received March 27th, 1979) 145

Reacti-Gel™

A MAJOR ADVANCE IN ACTIVATED MATRIX TECHNOLOGY



Carbonyldiimidazole Activated Polysaccharide Matrix

IMMOBILIZE:

- ✳ Ligands
- ✳ Leashes
- ✳ Hormones
- ✳ Proteins

REACTI•GEL (6X)

CDI Activated
Crosslinked 6% Agarose
(Stabilized in Acetone)

- ✳ Efficient Coupling of N-Nucleophiles
- ✳ Resulting Linkage is Devoid of Charge
- ✳ Mild Coupling Conditions

REACTI-GEL (6X): Carbonyl diimidazole Activated 6% Agarose, crosslinked $150/50 ml gel
Supplied stabilized in an acetone slurry. Agarose wet bead diameter; 40-210 µm. Activation level: 20-25 µM/ml gel. A practical guide to the use of REACTI-GEL (6X) accompanies the product.

PIERCE

PIERCE CHEMICAL COMPANY/Box 117/Rockford, Illinois 61105 U.S.A./Tele. 815-968-0747, 800-435-2960

Dictionary of Particle Technology

English-German German-English

by K. LESCHONSKI, *Technische Universität Clausthal, West Germany, and* F. T. C. CARTER, *Loughborough University of Technology, England*

Although particle technology is a relatively new science, its activities influence many areas of modern life. The properties of powders and particle dispersions in liquids and gases and the application of this knowledge to process operations has given the science of particle technology a broad scope - from the handling of bulk powders to air filtration in clean rooms, from particle production to both liquid and gaseous effluent control.

To give this new area of scientific vocabulary the special coverage it needs, here is Elsevier's **Dictionary of Particle Technology.** Although much of the research and subsequent applications in the field have been carried out, and thoroughly documented, in German and Anglo-Saxon countries, existing technical dictionaries fail to give adequate coverage of the vocabulary involved. Elsevier's **Dictionary of Particle Technology** concentrates, therefore, on comparative English and German terminology. For ease of reference and to avoid confusion for the user with only a basic knowledge of English or German, the compilers have limited the translation of each word to one equivalent in the other language, i.e. the one most commonly used in particle technology.

This is the only specialist English/German dictionary available for workers in this field and it will be an invaluable aid to the rapid translation of papers and books published in these languages.

1978 x + 286 pages US $49.00 / Dfl. 110.00 ISBN 0-444-41746-X

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.

Cellulose ion exchangers should be spherical too

DEAE-Sephacel® is

DEAE-**Sephacel**®
Total capacity: 1.4 meq/g
Wet diameter: 40–160 μm

The spherical form of DEAE-Sephacel gives you easy, uniform packing without the generation of fines. It improves your results because it reduces zone broadening and ensures uniform, high flow rates. DEAE-Sephacel is reproducible from batch-to-batch.

No precycling is needed because DEAE-Sephacel is ready swollen. Its controlled macroporous structure, which is almost completely independent of pH and ionic strength, gives high protein capacities and excellent recoveries.

DEAE-Sephacel is the only spherical cellulose ion exchanger.

Pharmacia Fine Chemicals AB
Box 175
S-751 04 Uppsala 1
Sweden

ref no 418

Recent Developments in Chromatography and Electrophoresis

Proceedings of the 9th International Symposium on Chromatography and Electrophoresis, Riva del Garda, 15-17 May, 1978

edited by **A. FRIGERIO** and **L. RENOZ**

CHROMATOGRAPHY SYMPOSIA SERIES, Volume 1

The symposium was organized by the Italian Group for Mass Spectrometry in Biochemistry and Medicine and the Belgian and Italian Societies for Pharmaceutical Sciences. This volume, as a result, comprises 34 papers presented at the symposium by specialists in various branches of chromatography and electrophoresis.

The proceedings, providing general reviews on chromatography and electrophoresis, cover a wide range of potential applications of these techniques. Of particular interest are the studies concerning identification of drugs, drug metabolites and pollutants, whilst other investigations are concerned with the identification of endogenous metabolites in living organisms. In addition, the medical applications of chromatography and electrophoresis for diagnostic purposes are presented. These techniques will undoubtedly become a necessary tool in every major hospital.

This work, reflecting current developments in the use of chromatography and electrophoresis, will be of value to research workers in chemistry, biochemistry, medicine, toxicology, drug metabolism, forensic science, clinical chemistry and pollution studies.

March 1979 x + 358 pages US $58.50/Dfl. 120.00 ISBN 0-444-41785-0

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

The Dutch guilder price is definitive. US $ prices are subject to exchange rate fluctuations.

7135

A new dimension in GC: the Packard 430

The new Packard 430 combines the fully programmable 429 gas chromatograph, with its powerful Multi Dimensional Switching System (MDSS) module, with a dual channel integrating and reporting system. The result is a gas chromatograph which combines tremendous analytical flexibility with powerful data handling to give you a final analysis report at the touch of a button. And all in one cabinet.

Simultaneous DUAL channel data reduction and reporting
Full integration and reporting of two independent channels is achieved with the use of only 5 extra keys; results are produced via a built-in integrator and printer. Optimum integration settings are automatically selected by the instrument itself, thus saving valuable time. Integrator and GC settings are also listed at request, while a bright display can report all set and sensed values. This visual check on entered data minimizes the chance of incorrect data entry.

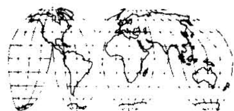
Ideal for Multi-Dimensional Switching Systems (MDSS)
The dual channel data reduction system is ideally suited to the MDSS module*. Increased analytical information is obtained by incorporation of fixed analytical routines such as solvent flushing, backflushing and sample transfer in the microprocessor. It enables the chromatographer to optimize an analysis with regard to identification certainty and analysis time.

* optional

Total analytical flexibility
Unique design features such as the Analytical Module, truly modular plug-in flow design combined with the possibility of storing 18 complete programs of 15 time based instructions each, allow each individual chemist to tailor the system to his own requirements.

Write or call your nearest Packard office for more detailed information. Our GC sales specialists will be glad to tell you more about this remarkable system.

Packard: the professionals in gas chromatography



Packard

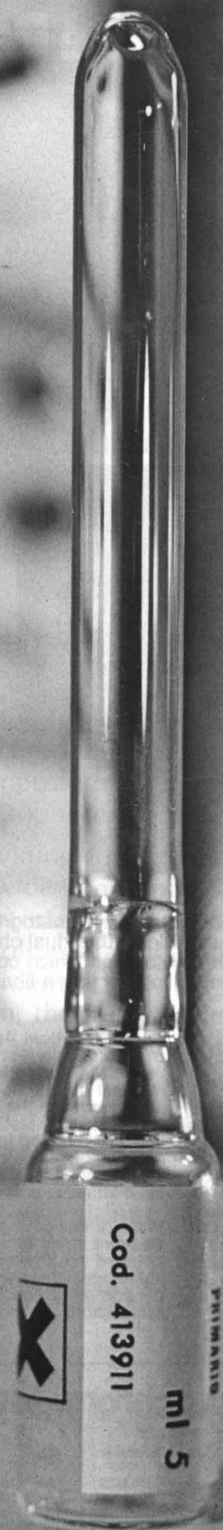
PACKARD-BECKER B.V.
VULCANUSWEG [illegible], P.O. BOX 519, DELFT, NETHERLANDS

PACKARD INSTRUMENT INTERNATIONAL S.A.
RENGGERSTRASSE [illegible] ZURICH, SWITZERLAND

PACKARD INSTRUMENT COMPANY, INC.
2200 WARRENVILLE RD, DOWNERS GROVE, ILL 60515

SUBSIDIARIES OF AMBAC INDUSTRIES, INC.

ref no 439



STANDARD FOR GAS CHROMATOGRAPH

A wide range, constantly being increased, of pure substances suitable for use as Standards for Gas Chromatography, includin

- **Hydrocarbons (paraffins, isoparaffins, cycloparaffins aromatics, olefins, cycloolefins and diolefins)**
- **Fatty acids and methyl esters of fatty acids**
- **Alcohols, including fatty alcohols**
- **Fatty acid anhydrides**

Their purities are guaranteed by a gas-chromatogram included in each package, which gives the conditions used in our laboratories for these control analyses. When using the low boiling hydrocarbons it is advisable to take the precaution of cooling the ampoule to about 0°C before opening.

ARMITALIA CARLO ERBA*

ALYTICAL DIVISION / REAGENTS / 20159 Milano / Via C. Imbonati 24

JOURNAL OF CHROMATOGRAPHY

VOL. 176 (1979)

JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

EDITOR
MICHAEL LEDERER (Rome)

ASSOCIATE EDITOR
K. MACEK (Prague)

EDITORIAL BOARD
W. A. Aue (Halifax), V. G. Berezkin (Moscow), V. Betina (Bratislava), A. Bevenue (Honolulu, Hawaii), P. Boulanger (Lille), A. A. Boulton (Saskatoon), G. P. Cartoni (Rome), G. Duyckaerts (Liège), L. Fishbein (Jefferson, Ark.), A. Frigerio (Milan), C. W. Gehrke (Columbia, Mo.), E. Gil-Av (Rehovot), G. Guiochon (Palaiseau), I. M. Hais (Hradec Králové), E. Heftmann (Berkeley, Calif.), S. Hjertén (Uppsala), E. C. Horning (Houston, Texas), Cs. Horváth (New Haven, Conn.), J. F. K. Huber (Vienna), A. T. James (Sharnbrook), J. Janák (Brno), E. sz. Kováts (Lausanne), K. A. Kraus (Oak Ridge, Tenn.), E. Lederer (Gif-sur-Yvette), A. Liberti (Rome), H. M. McNair (Blacksburg, Va.), Y. Marcus (Jerusalem), G. B. Marini-Bettòlo (Rome), Č. Michalec (Prague), R. Neher (Basel), G. Nickless (Bristol), J. Novák (Brno), N. A. Parris (Wilmington, Del.), P. G. Righetti (Milan), O. Samuelson (Göteborg), G.-M. Schwab (Munich), G. Semenza (Zürich), L. R. Snyder (Tarrytown, N.Y.), A. Zlatkis (Houston, Texas)

EDITORS, BIBLIOGRAPHY SECTION
K. Macek (Prague), J. Janák (Brno), Z. Deyl (Prague)

EDITOR, BOOK REVIEW SECTION
R. Amos (Abingdon)

EDITOR, NEWS SECTION
J. F. K. Huber (Vienna)

COORDINATING EDITOR, DATA SECTION
J. Gasparič (Hradec Králové)

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

J. Chromatogr., Vol. 176 (1979)

© ELSEVIER SCIENTIFIC PUBLISHING COMPANY — 1979

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the publisher, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Submission of an article for publication implies the transfer of the copyright from the author to the publisher and is also understood to imply that the article is not being considered for publication elsewhere.

Submission to this journal of a paper entails the author's 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.

Printed in The Netherlands

Journal of Chromatography, 176 (1979) 1–9
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,889

PARAMETER STUDY OF A HYDROGEN ATMOSPHERE FLAME IONIZATION DETECTOR

J. E. ROBERTS and H. H. HILL, Jr.*
Department of Chemistry, Washington State University, Pullman, Wash. 99164 (U.S.A.)
(Received February 2nd 1979)

SUMMARY

A hydrogen atmosphere flame ionization detector was constructed and operated with a direct injection technique in order to investigate detector parameters that have not previously been characterized. The optimum concentration of silane added to the hydrogen atmosphere was found to be dependent on the height of the collector electrode. At higher electrode heights, the required concentration of silane decreased. In this study, a collector height of 70 mm and a silane mixing ratio of 4 ppm were chosen as the best conditions. Also, optimal response was found when the nitrogen/oxygen ratio was *ca.* 1 and when the hydrogen flow was 3 l/min.

Several observed phenomena provided indirect evidence of the detector's mechanism. The interdependence of silane doping and electrode height supports a charge transfer process within the flame as the source of enhanced metal response. Negative peaks, observed when the off-center electrode is close to the flame, are explained as the formation of ions, via charge transfer reactions, that have reduced ionic mobilities and are more inefficiently collected by the electrode than parent ions.

INTRODUCTION

Several years ago a flame phenomenon was reported in which organometallic compounds were seen to produce an ionization response three to five orders of magnitude greater than that observed for other organic compounds[1]. This flame was developed into a sensitive and selective gas chromatographic (GC) detector in which compounds such as aluminum hexafluoroacetylacetonate, ferrocene, tetraethyllead and tetraethyltin were selectively detected at picogram and sub-picogram levels[2–4].

Although response was a result of enhanced ionization in a flame similar to a standard flame ionization detector (FID), operating conditions are substantially different. The flame of a hydrogen atmosphere flame ionization detector (HAFID) is fed with oxygen-enriched air and burns in a hydrogen atmosphere, doped with a small amount of silane. Ions are collected with a negatively polarized electrode located 5–7 cm above the flame.

* To whom correspondence should be addressed.

Possible mechanisms have been discussed elsewhere[4]. Here, it need only be clear that burning a flame in a hydrogen atmosphere doped with silane and then collecting ions several centimeters from this flame are novel conditions for efficient ion detection. Previous studies have concentrated on these more notable parameters at the expense of careful documentation of the effects that gas flows have on response.

In the past, operating conditions for the detector's gas flows were selected for reasons other than the best analytical response. When this enhanced ionization phenomenon was first observed, flow-rates were optimized for a photometric detector to which the ionization response served as an auxillary detection mode. They were 50 ml/min of nitrogen GC carrier gas, 70 ml/min of air, 96 ml/min of oxygen and 830 ml/min of hydrogen[1,2]. Flow conditions were modified in a second detector, designed specifically as an HAFID. They were 40 ml/min of nitrogen GC carrier gas, 160 ml/min of air, 85 ml/min of oxygen and 950 ml/min of hydrogen. Their selection was based on the best combination for ignition of the flame rather than on the best response[3]. Later, in order to eliminate excessive baseline oscillations that occurred in certain detector geometries, these flows were adjusted to 40 ml/min of nitrogen carrier gas, 100 ml/min of nitrogen purge gas, 125 ml/min of oxygen and 1600 ml/min of hydrogen[3,4].

Throughout development several qualitative statements about flow parameters have been made. It was often noted that an increase in oxygen increased the response of metal compounds while it decreased the response of hydrocarbon standards[1–4] and that minor changes in hydrogen flow had little effect on response except at low flows where hydrocarbon response increased[3]. Nitrogen was considered only to affect the oxygen concentration. Unfortunately, much of the earlier gas flow studies were completed before the necessity of silane doping was discovered[4], and silane concentrations were not closely controlled during these investigations.

Application of the HAFID to organotin compounds appears particularly promising because of the ease with which these compounds can be analyzed by gas chromatography and because the increasing use of organotins as antifungal agents has produced a need for their quantitation at low concentration levels. In this study, a detector was designed to investigate operating conditions of the HAFID related specifically to organotin detection with careful control of the level of silane present in the hydrogen atmosphere.

EXPERIMENTAL

Detector design

The detector, shown in Fig. 1, consists of a base, a jet, a sleeve and a cap. The base was machined from a block of stainless steel 316 which provided passages for hydrogen, oxygen and air nitrogen. Baffels where hydrogen entered the detector served to diffuse streaming effects of high flow-rates. Nitrogen and oxygen were introduced through a stainless-steel jet (20 mm $\times$ 1/8 in. O.D. $\times$ 1 mm I.D.) that was swaged to the detector's base so that an oxygen–nitrogen fed flame burned at its exit in a hydrogen atmosphere. This atmosphere was contained by a stainless steel 316 sleeve (26 mm I.D.). A number of sleeves varying in length were threaded at each end so that one end of a sleeve could be securely screwed into the detector's base and the other fitted with a cap containing a collecting electrode, glow plug ignitor and numerous exhaust vents. A male fitting BNC chassis connector served as the collecting

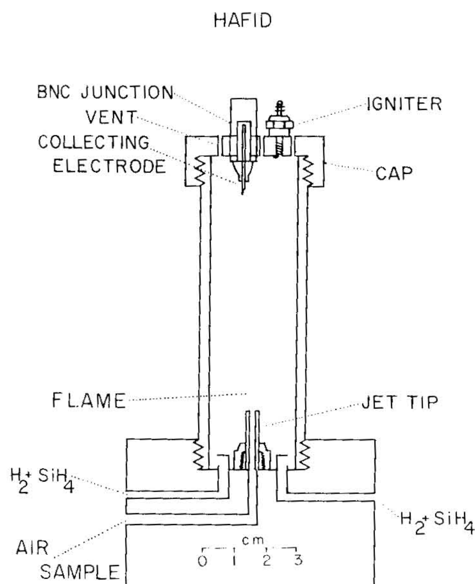


Fig. 1. Schematic of a hydrogen flame ionization detector.

electrode and was polarized with −90 V from a Model No. 490 Eveready battery which was floated between the electrode and a Keithly 427 Micro-Micro Ampmeter. A Sargent-Welch SKR strip chart recorder traced the signal.

Flame ignition

Although large amounts of hydrogen are used in this detector, flame ignition is safe, reproducible and even quiet if the proper procedure is followed. With the hydrogen adjusted to *ca.* 1500 ml/min and turned off by a simple on/off valve, the oxygen and air were introduced through the jet tip at rates of 135 ml/min and 150 ml/min, respectively. After purging, the glow plug ignitor was turned on followed by hydrogen introduction. A low, muffled sound indicated ignition while a loud pop usually meant flows were not adjusted properly or residual hydrogen from previous runs had not been sufficiently swept from the detector. When the flame has been properly ignited, the tip of the stainless-steel jet glows red-orange and water vapor condenses readily on cold objects held in the exiting gases.

Silane doping

In the past, doping the hydrogen atmosphere with silicon compounds has been accomplished from bleed of a polysiloxane column, silicon rubber in the heated detector, vapors of tetramethylsilane, or gaseous silane[4]. Silane appears to be the best of these doping agents but is difficult to accurately control at the low flows required (*ca.* 7 μl/min) when pure silane is mixed with hydrogen. For these studies a tank of hydrogen was purchased, premixed with silane at the 100 ppm level ($\pm 10\%$), from AIRCO, Rare and Specicialty Gases (Santa Clara, Calif., U.S.A.). It was a simple procedure to further dilute this mixture by combining gas streams to produce a hy-

drogen atmosphere in which the silane mixing ratio could be controlled down to 1 ppm.

Sample introduction

Tetramethyltin was contained as a vaporun der nitrogen in a stainless-steel tank whose exit was connected to one port of a six-port zero-volume valve (Valco Instrument Co., Houston, Texas, U.S.A.). With the tank pressurized at 5 atm the sample was passed through a 0.88-cm^3 sample loop that could be flushed into the detector with a turn of the valve.

The standard was prepared by injecting 2.5 μl of tetramethyltin into a prewashed, prebaked and evacuated 0.5-l sample tank. The tank was then pressurized to 5 atm with nitrogen. After thoroughly mixing the tank's contents, the pressure was reduced to 1 atm and then repressurized to 5 atm with pure nitrogen, producing a standard concentration of 0.26 μg/ml of tetramethyltin. Each 0.88-cm^3 injection resulted in a 0.23-μg injection into the flame. This value was confirmed by injecting a sample into a gas chromatograph and comparing its response with standards. Injections of 0.23 μg of tetramethyltin were chosen because this quantity produces a response that just saturates the optimized detector while permitting studies of non-optimum regions as well.

Reproducibility of this sampling system was established by comparing peak height responses of injections made from tank pressures varying from 5 atm to 1 atm. The tank was then emptied, flushed several times with nitrogen and refilled with a fresh sample. An injection from this new sample was compared to previous ones. All responses agreed within 5%.

Experiments

Oxygen/nitrogen ratio. The optimum ratio of oxygen flow to air flow through the jet tip was established by holding the total flow of carrier gas nitrogen (20 ml/min), air and oxygen constant at 305 ml/min while varying the nitrogen from 7% to 81%. The silane was maintained at a constant mixing ratio of 9 ppm in a 1500 ml/min hydrogen flow. Ions were collected with a −90 V, electrode, 50 mm above the jet tip.

Silane doping vs. electrode height. Investigation of optimum silane doping conditions as a function of collecting electrode height was accomplished by varying the silane from 1 ppm to 11 ppm for electrode heights of 10, 30, 50, 70, 90 and 110 mm. Throughout this study gas flow-rates were held constant at 120 ml/min of oxygen, 165 ml/min of air and 1500 ml/min of hydrogen.

Hydrogen flow. With a silane concentration of 4 ppm and an electrode height of 70 mm, the hydrogen gas flow was varied from 1 to 4 l/min. For each hydrogen flow investigated, silane was adjusted so that its mixing ratio remained at 4 ppm. The oxygen and air flows were maintained at 120 ml/min and 165 ml/min, respectively.

RESULTS AND DISCUSSION

Nitrogen

Although in earlier studies nitrogen has been added to the oxygen stream, its effect on response has never been documented. Fig. 2 illustrates the effect when the total oxygen plus nitrogen flow is held constant at 305 ml/min and nitrogen is

progressively introduced into the oxygen stream by increasing the air/oxygen ratio. When no air was added to oxygen, 20 ml/min of carrier gas was the only source of nitrogen in the flame, resulting in *ca.* 7% nitrogen. When air was the only source of oxygen, 285 ml/min of air plus 20 ml/min of nitrogen carrier gave a maximum nitrogen content of 81%. From Fig. 2 it is clear that when either oxygen or air is used alone, response is diminished. Air must be enriched with oxygen to produce the maximum response. Optimal oxygen and nitrogen values were found to be 50% and 50%, respectively, corresponding to flows of 20 ml/min for nitrogen carrier gas, 165 ml/min for air and 120 ml/min for oxygen.

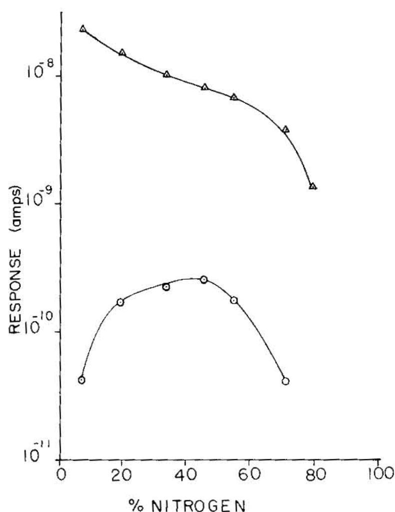


Fig. 2. Peak height response of tetramethyltin as a function of % nitrogen in oxygen with the total flow held constant at 305 ml/min. Hydrogen doped with 4 ppm SiH_4 was held constant at 1500 ml/min. Electrode = − 90 V at a height of 70 mm. Flow-rate of N_2 carrier = 20 ml/min. △, Flame background; ○, tetramethyltin response.

Silane doping vs. electrode height

The importance of high electrode heights and silane doping of the hydrogen atmosphere to the HAFID has been previously reported but an interdependent relationship between these two parameters as shown in Fig. 3 is truly surprising. The response in ampère for each injection of tetramethyltin was measured at the peak maximum and plotted as a function of ppm silane in hydrogen. Each curve represents a different collecting electrode height. The response curve for the collecting electrode height of 50 mm compares favorably with that from ref. 4 in which the peak height response dependence of 10 ng of tetrabutyltin with varying silane mixing ratios was demonstrated at an electrode height of 50 mm. From Fig. 3 the optimum silane mixing ratio is seen to be 9 ppm compared to the value reported earlier of 5 ppm. When holding this silane level at 9 ppm it is clear from Fig. 3 that varying the electrode height would show 50 mm to be the optimum choice. Yet, it is also clear from the graph that a more sensitive response can be obtained for tetramethyltin with an electrode height of 70 mm if the doping concentration of silane is lowered to 5 ppm.

In fact, each electrode height studied has a different optimal silane doping

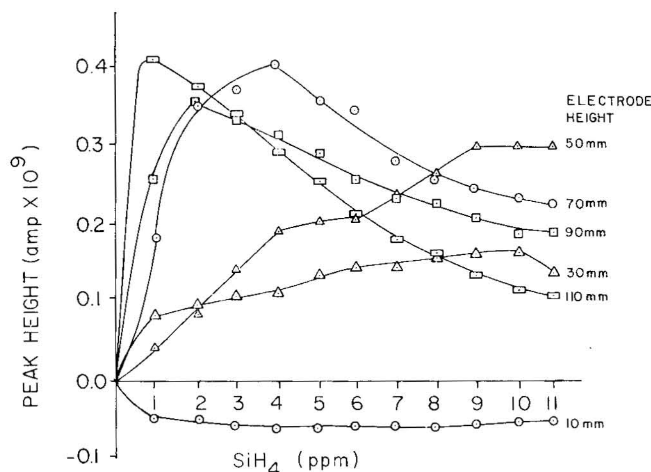


Fig. 3. Curves of silane doping *vs.* electrode height. Gas flow-rates in ml/min: hydrogen, 1500; oxygen, 120; air, 165; nitrogen carrier, 20.

level. Fig. 4 plots the amount of silane required for the maximum response at each electrode height. Higher electrodes require less silane in the atmosphere for best response but, in each case, when no silane is present, the response is immeasurable.

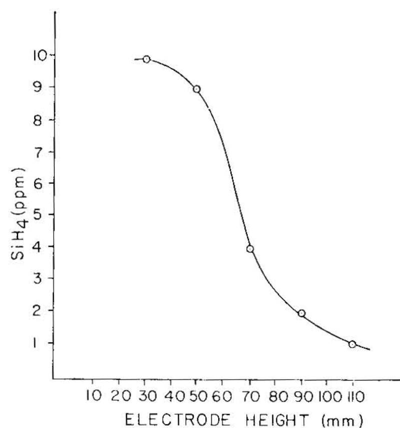


Fig. 4. Optimum silane mixing ratios for each electrode height in Fig. 3.

Speculation about the mechanism of the HAFID response has suggested that a charge transfer process within the flame or in the afterglow region is responsible for the enhanced ionization of organometallic compounds[4]. These data support that postulate. It suggests that SiH_4 is not involved in the initial ionization process since the production of ions by reaction of neutral molecules or fragments with SiH_4 in the flame region would be independent of the height of the electrode. However, the inverse relationship of these two parameters may indicate that SiH_4 is involved with an ion–molecule reaction. When the electrode is closer to the flame and the electric field is greater, ions spend less time in the reaction zone. If SiH_4 is the molecular

portion of an important ion–molecule reaction, then it would seem reasonable that an increase in SiH_4 would be required to insure optimal interaction when the residence time of the ion is decreased.

For application, it is important to realize that sensitivity may be enhanced at higher electrode heights by reducing the amount of SiH_4 in the atmosphere. Furthermore, hydrocarbon response, background ionization and noise decrease with increasing electrode height, making it desirable to operate at the highest electrode height at which the sensitivity required for analysis can be obtained.

Negative peaks

Contrary to results reported earlier where all electrode heights produced positive peaks[4], the response of this detector at an electrode height of 10 mm produced negative peaks (a decrease in ionization) at all silane concentrations studied. An explanation of this phenomenon can be based on the specific design of this detector. The collecting electrode of the detector described in ref. 4 was a platinum loop centered above the jet tip while the collector of this work was a pin electrode positioned at some angle θ) from the jet tip (see Fig. 5). At electrode heights of 30 mm or more, θ is small and has little effect on the response since as the linear velocity decreases above the flame, ions are more easily attracted to the electrode's negative field. At 10 mm, however, the angle between the jet tip and the collector is sufficiently large to permit the competition between mass flow and ion flow.

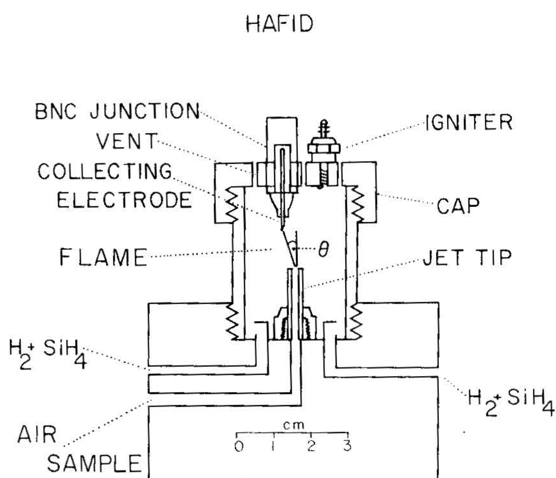


Fig. 5. Schematic of HAFID with electrode height of 10 mm. θ is the angle of ion drift for collection at a "pin" electrode.

The mass flow velocity of an ion or molecule emerging from the flame can be approximated to be 650 cm/sec from the flow-rate (305 ml/min) and the diameter of the jet tip (1 mm). The ion drift velocity as a result of the electric field intensity can be approximated from a rearrangement of the "reduced mobility" (K_0) equation[5]

$$V_d = K_0 E \left(\frac{760}{P}\right) \left(\frac{T}{273}\right)$$

where K_0 is a constant for a given ion species called the "reduced mobility", P is the gas pressure in Torr, T is the gas temperature in degrees Kelvin, E is the electric field intensity and V_d is the ionic drift velocity. Assuming a constant pressure of 700 Torr within the detector, a temperature of 700 °C above the flame, an electric field of 90 V/cm and a K_0 value of 2 $cm^2/sec \cdot V$ (typical[6] values range from 1 to 3 $cm^2/sec \cdot V$), the ion drift velocity can be calculated to be *ca.* 700 cm/sec. Although these calculations are crude since conditions such as electric field intensity and gas temperature are not homogeneous within the HAFID, they point out that ions formed in the flame may have two competing modes of transportation, a coulombic attraction to the electrode where the ion will be reduced to produce a measurable current and a mass flow of the ion past the electrode to the detector's cap.

Negative peaks that occur when tetramethyltin is burned in the flame can be explained by the formation of ions, via charge transfer reactions, with reduced ionic mobilities (indicating a higher molecular weight) which are more inefficiently collected by the electrode than are the ions responsible for the background current.

Hydrogen

The effect that various hydrogen flow-rates have on response has not previously been reported except for cursory investigations[4] where it was not clear whether changes in response resulted from the varying hydrogen flow or the varying silane concentration. Fig. 6 shows the results of changing hydrogen flows and silane flows so that the mixing ratio remains constant at each flow tested. With a silane concentration of 4 ppm and an electrode height of 70 mm the response for tetramethyltin increased with increasing hydrogen flows to a maximum of 3.0 l/min. Since these high flows limit the practical potential of this detector, future developmental work will be aimed at redesigning the detector to reduce this hazardous hydrogen flow while maintaining the increased sensitivity.

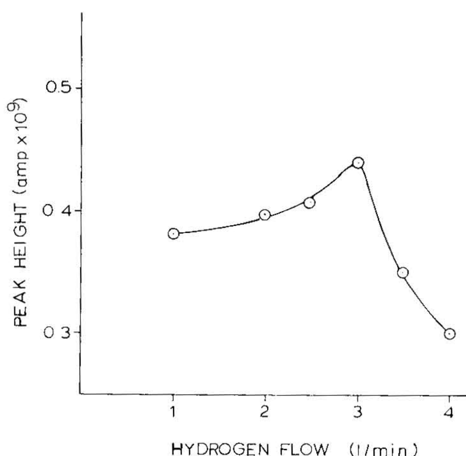


Fig. 6. Peak height response of tetramethyltin at various hydrogen flow-rates. The SiH_4 mixing ratio held constant at 4 ppm. Electrode height: 70 mm. Gas flows in ml/min: oxygen, 120; air, 165; nitrogen carrier, 20.

CONCLUSION

This study has served to document several novel parameters associated with fine tuning procedures of a metal-sensitive hydrogen atmosphere flame ionization detector. While tetramethyltin was the only compound investigated, organotin response is known to behave similarly to a variety of organometallic compounds[4], indicating that parameters discussed here are also important for the application of this detection system to the determination of other volatile metal compounds.

The optimal values that have been established in this study are not necessarily expected to be identical for other hydrogen atmosphere flame systems which differ in detector geometry. Nevertheless, this study has identified operating parameters which are important to optimize to obtain the best sensitivity from the system. Interdependency of two parameters, silane doping and electrode height, has been demonstrated which leads to concern over the interrelationship of other detector parameters. Such a complicated optimization procedure is best left to the analyst who is developing a specific analytical procedure. Total optimization would require collected consideration of the oxygen/nitrogen flow ratio and rates, the electrode height and potential, the hydrogen flow-rate and the silane doping level.

Similar to the highly successful electron capture detector which is seldom operated at its optimum, accurate adjustment of all parameters to obtain absolute optimization of the HAFID is not required for its use as a sensitive and selective metal-sensing device. Following guidelines for operation presented in this and related papers will allow the facile conversion of a commercial flame ionization detector into a selective ionization detector that is practical for many organometallic determinations in the sub-nanogram and picogram range.

ACKNOWLEDGEMENTS

Acknowledgement is made to the Donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research under Grant No. 9920-G3,5 and to the National Science Foundation for partial support under Grant No. CHE 77-25743. The authors also thank T. Kumar for his help with this project.

REFERENCES

1 W. A. Aue and H. H. Hill, Jr., *J. Chromatogr.*, 74 (1972) 319.
2 W. A. Aue and H. H. Hill, Jr., *Anal. Chem.*, 45 (1973) 729.
3 H. H. Hill, Jr. and W. A. Aue, *J. Chromatogr. Sci.*, 12 (1974) 541.
4 H. H. Hill, Jr. and W. A. Aue, *J. Chromatogr.*, 122 (1976) 515.
5 E. W. McDaniel and E. A. Mason, *The Mobility and Diffusion of Ions in Gases*, Wiley, New York, 1973, p. 6.
6 F. W. Karasek, *Anal. Chem.*, 45 (1974) 710A.

Journal of Chromatography, 176 (1979) 11–18
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,880

REGENERABLE AFFINITY CHROMATOGRAPHY SUPPORT

V. K. JOSHI and K. M. SHAHANI

Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, Nebr. 68583 (U.S.A.)

A. KILARA

Department of Food Science, Pennsylvania State University, University Park, Pa. 16802 (U.S.A.)

and

FRED W. WAGNER*

Laboratory of Agricultural Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebr. 68583 (U.S.A.)

(First received December 28th, 1978; revised manuscript received March 23rd, 1979)

SUMMARY

A derivative of Sepharose 4B, *p*-(N-acetyl-L-tyrosineazo)benzamidoethyl Sepharose 4B, was synthesized and used for the selective immobilization of proteins. The azo bond of this derivative can be reduced to liberate *p*-aminobenzamidoethyl Sepharose 4B, which can be diazotized and recoupled to N-acetyl-L-tyrosine and subsequently to a fresh preparation of protein. This regenerable affinity chromatography support was regenerated successively through five cycles without detectable loss of functional groups.

INTRODUCTION

The use of immobilized proteins has become common, and a variety of methods exist for coupling proteins to solid supports. In our experience, variations in the method described by Cuatrecasas and Parikh[1] have been most advantageous as proteins are coupled principally via α-amino groups, minimizing the number of points of chemical attachment[1]. This method has been shown to have a minimal influence on biological activity of a variety of proteins immobilized in our laboratories, including lactase[2], papain[3], lipase[3], antibodies[4], leghemoglobin**, mitochondrial ATPase[5] and a mixture of enzymes co-immobilized to a single support[6].

Application of immobilized proteins and enzymes to industrial processes has received considerable attention recently[7]. One of the principal problems of using large-scale immobilized proteins is the high cost of the solid support, which is discarded after the biological activity of the ligand is lost.

---

* To whom all communications should be addressed.

** Unpublished data.

We have synthesized a novel spacer arm on a solid support for immobilizing enzymes and proteins, which offers all the advantages of the method of Cuatrecasas and Parikh[1], but also can be regenerated to remove denatured protein and reused to immobilize native protein of the same or different identity.

EXPERIMENTAL

Materials

Sepharose 4B was purchased from Pharmacia (Piscataway, N.J., U.S.A.). Special chemicals used included N-hydroxysuccinimide, dicylcohexylcarbodiimide (Eastman, Rochester, N.Y., U.S.A.), *p*-nitrobenzoyl chloride (Aldrich, Milwaukee, Wisc., U.S.A.), L-valine methyl ester, N-acetyl-L-tyrosine (Sigma, St. Louis, Mo., U.S.A.) and L-[^{14}C]valine (Schwartz/Mann, Orangeburg, N.Y., U.S.A.).

*Preparation of RACS**

PABS was prepared exactly by the method of Cuatrecasas[8] using 250 mg of cyanogen bromide per ml of packed gel. Briefly, cyanogen bromide-activated Sepharose 4B was allowed to react with ethylenediamine to form aminoethyl Sepharose 4B. The latter product was then allowed to react with an equal volume of 0.07 *M* *p*-nitrobenzoylazide in 40% dimethylformamide and 0.2 *M* sodium borate buffer pH 9.3 at room temperature for 1 h. The product was thoroughly washed with 50% dimethylformamide in the same buffer, then with 0.2 *M* sodium borate, pH 8.5, and incubated in 0.1 *M* sodium dithionite in 0.2 *M* sodium borate for 1 h to reduce the *p*-nitrobenzamidoethyl Sepharose 4B.

p-Nitrobenzoylazide was prepared by slowly adding 0.7 g of sodium azide dissolved in 2 ml of ice-cold water to an ice-cold solution of *p*-nitrobenzoyl chloride (2.45 g in 25 ml of acetone). The reaction was kept below 4° by incubating the solution of a bath of melting ice for 15 min. The product, *p*-nitrobenzoylazide, was obtained by adding 50 ml of cold distilled water to the reaction mixture. It was filtered and washed with water, then dried over P_2O_5. The yield of the product was 76%.

PABS (150 ml packed gel) was suspended in 250 ml of 0.1 *N* HCl and chilled to 4°, then 1.4 g of $NaNO_2$ was added and diazotization was allowed to proceed for 7 min[8]. The diazotized resin was filtered and washed with cold 0.1 *N* HCl. The filter cake was slowly added to 150 ml of 0.1 *M* $NaHCO_3$, pH 9.3, containing 10 μmol of N-acetyl-L-tyrosine per milliliter of packed gel. The azo coupling reaction was allowed to proceed for 3 h at room temperature. As the reaction progressed the resin became deep red. The reaction product, RACS, was washed with water and then dioxane, and stored at room temperature in dioxane until used.

Preparation of L-[^{14}C]*valine methyl ester*

L-[^{14}C]Valine (1.4 ml, 70 μCi, sp. act. 260 mCi/mol) was dried for 16 h over

* Abbreviations: RACS = regenerable affinity chromatography support; PABS = *p*-aminobenzamidoethyl Sepharose UB; ONPG = *o*-nitrophenyl-β-D-galactopyranoside; DCC = dicyclohexylcarbodiimide.

P_2O_5 under reduced pressure. The dried valine was dissolved in 1 ml of hot methanol and then diluted to 2 ml with diethyl ether. Methylation was performed using diazomethane, by the procedure of Schlenk and Gellerman[9]. The product, L-[^{14}C]valine methyl ester, was dissolved in methanol and added to 1.5 g of L-valine methyl ester in 5 ml of methanol. L-[^{14}C]Valine methyl ester of sp. act. 6.5 mCi/mol was crystallized by the slow addition of diethyl ether. The crystals were collected, washed with diethyl ether and dried (yield 99.8%). Thin-layer chromatography of the product gave one ninhydrin positive spot using methanol as the solvent, which resolves the methyl ester from the free acid.

Reduction and regeneration of RACS

The column containing the desired amount of RACS was washed at room temperature with 10 volumes of 0.1 *M* sodium dithionite in 0.2 *M* sodium borate buffer, pH 9.0. The color due to the azo bond disappeared after *ca.* 5 volumes of buffer had passed through the resin. The column was then washed with distilled water to remove excess dithionite. Reduction reconverted RACS into PABS. To regenerate the azo bond to N-acetyl-L-tyrosine, the resin was diazotized and treated as described above. The initial resin (150 ml packed gel) was recycled in this manner five times and a 20 ml packed gel sample was saved after each cycle.

Determination of available binding sites

L-[^{14}C]Valine methyl ester was coupled to RACS using DCC. Three ml of packed gel were suspended in 9 ml 1,4-dioxane containing 0.2 g of DCC and 30 μmol of L-[^{14}C]valine methyl ester. The reaction was allowed to proceed for 90 min at room temperature. The resin was washed in 2 volumes of dioxane, followed by 2 volumes of methanol and finally 4 volumes of dioxane. The product was dried and aliquots of the resin were assayed for radioactivity. Other methods of coupling used DCC in dimethylformamide and 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide, but these agents were not as effective as DCC in 1,4-dioxane.

To study the effect of reaction time on the quantitative attachment of L-[^{14}C]valine methyl ester, aliquots were withdrawn from the reaction mixture at 20, 40, 70, 90, 120, 140 and 160 min using dioxane, as described above. They were washed, dried and assayed for radioactivity.

Coupling of Enzymes

Aliquots of RACS and recycled RACS were suspended in 2 volumes of dioxane and to them were added DCC and N-hydroxysuccinimide to bring their concentrations to 0.1 *M*. The reaction was allowed to proceed for 90 min and the resin was washed successively with dioxane, methanol and dioxane. The resin was air-dried for 5 min to evaporate residual dioxane then suspended in an equal volume of 0.1 *M* potassium phosphate buffer, pH 7.0, containing 40 mg of enzyme per milliliter of packed gel. The reaction was allowed to proceed for 16 h at 4° and then terminated by rendering the reaction mixture 0.2 *M* with glycine. The product was washed extensively with cold 0.1 *M* phosphate buffer, pH 7.0, until all surface-absorbed enzyme was eluted. Enzymes immobilized were lactase ('maxilact', Enzyme Development Corp., N.Y.), papain, beef heart mitochondrial ATPase and lipase. With the exception of the ATPase, which was a gift of Dr. Sheldon M. Schuster,

Department of Chemistry, University of Nebraska-Lincoln, these enzymes were obtained commercially.

Enzymes were assayed using ONPG as the substrate for lactase[3], casein as the substrate for papain[3], butter oil as the substrate for lipase[11] and ATP as the substrate for ATPase[5]. The amounts of enzyme immobilized were determined from the activity level of the resin and expressed as the corresponding weight of soluble enzyme. This correlation is justified and based on earlier work from these laboratories[2] which showed these procedures to yield similar coupling levels of lactase (0.5 mg/ml) based on the amino-acid composition of immobilized preparations. The soluble and immobilized forms of lactase were used to determine the pH optimum and the $K_{m(app)}$. The kinetic data were analyzed statistically by the method of Wilkinson[10].

RESULTS AND DISCUSSION

The structure of RACS and the chemical method used for its regeneration are shown in Fig. 1. The synthesis of the arm involved activation of Sepharose 4B and coupling of ethylenediamine, followed by reaction with *p*-nitrobenzoylazide. The *p*-nitrobenzamidoethyl Sepharose 4B was then reduced with dithionite to form PABS. Procedures for these syntheses are well documented[8]. The advantage of this arm is that it can be diazotized to form azo compounds with aromatic amines or phenolic compounds. Subsequent reduction of the azo bond regenerates PABS. The next section of the arm therefore had to be a phenolic or aromatic compound with a carboxylic acid function, which is necessary for the selective attachment of proteins primarily via α-amino groups[1]. N-Acetyl-L-tyrosine was selected, because it is inexpensive and readily available. On regeneration, its loss with the undesirable ligand is of no consequence (Fig. 1).

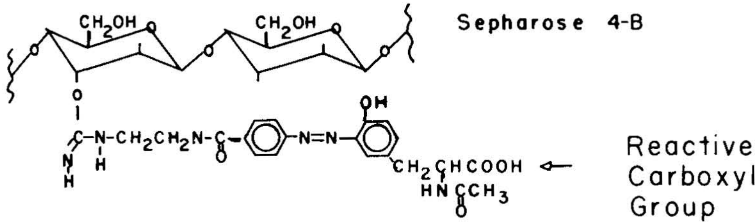
Resin containing *ca.* 2 μmol of N-acetyl-L-tyrosine per milliliter of packed gel was obtained by this synthesis, as evidenced by its ability to couple L-[^{14}C]valine methyl ester in the presence of DCC (Fig. 2). Other methods for coupling L-[^{14}C]valine methyl ester were not as effective as DCC in dioxane (Table I).

To test the efficacy of regeneration, 100 ml of RACS was packed into a column and washed with 0.1 *M* dithionite in 0.2 *M* sodium borate buffer, pH 9.0. Reduction of the arm could be followed visually as the loss of the red color of the resin due to to the presence of the azo linkage. The product, PABS, could be diazotized and recoupled to N-acetyl-L-tyrosine. Regenerated resin could then be used to couple L-valine methyl ester or protein. Table II shows the influence of recycling the resin on its ability to be activated and react with either L-valine methyl ester or "maxilact", a commercial preparation of lactase from *Saccharomyces lactis*. Recycling the resin five complete times did not affect its ability to react either with L-valine methyl ester in the presence of DCC or with lactase when the resin was activated with DCC and N-hydroxysuccinimide.

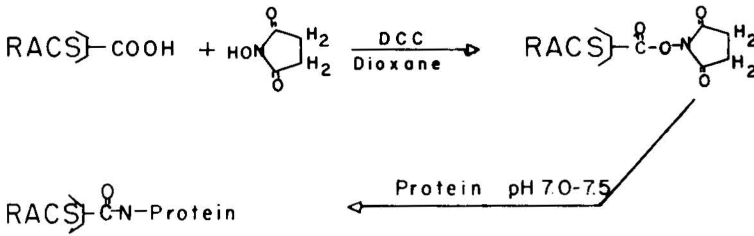
In earlier work[2] we have demonstrated that immobilizing lactase from *S. lactis* by the method of Cuatrecasas and Parikh[1] using succinylaminoethyl Sepharose 4B did not affect either the $K_{m(app)}$ or the pH optimum for the hydrolysis of ONPG. Use of RACS and this same coupling procedure yielded a resin containing *ca.* 0.35 mg of lactase bound per millilitre of packed RACS, regardless of the number of times the resin had been recycled. Fig. 3 shows the pH optimum and Lineweaver–

Burk plot for immobilized and soluble forms of lactase with ONPG used as substrate. Neither the pH optimum nor the $K_{m(app)}$ for the hydrolysis of ONPG was affected by the immobilization of lactase on RACS or by prior regeneration of the resin.

A Structure of RACS



B Immobilization of protein



C Regeneration

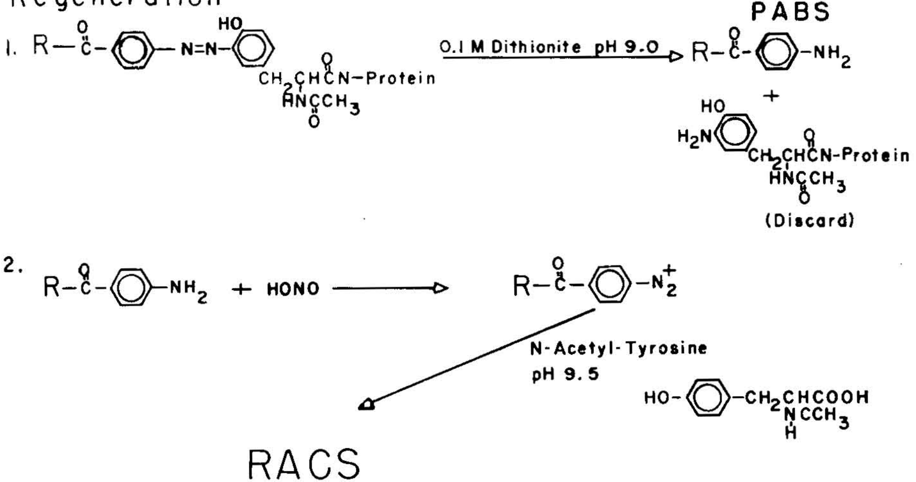


Fig. 1. Schematic representation of the regenerable affinity chromatography support (RACS). A, Structure of the arm; B, reactions involved to immobilize proteins[1]; C, reactions involved in removing denatured protein and adding new protein.

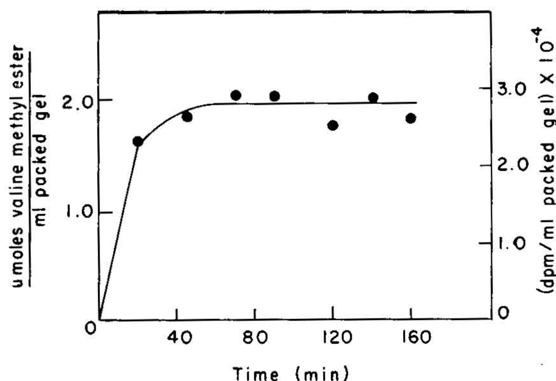


Fig. 2. Incorporation of L-[^{14}C]valine methyl ester into RACS. RACS (3 ml packed gel) was allowed to react in 6 ml of dioxane with 30 μmol of L-[^{14}C]valine methyl ester for the times shown. To stop the reaction, RACS was filtered and washed with dioxane, methanol and dioxane. To avoid variations in estimating the packed volume of RACS, the density of the packed gel was determined by weighing a dried sample of RACS from a known volume.

TABLE I

EFFECT OF THREE ACTIVATION PROCEDURES ON THE L-[^{14}C]VALINE METHYL ESTER REACTIVITY OF RACS

Activation procedure	*μmol L-[^{14}C]valine methyl ester per ml packed gel*
DCC in 1,4-dioxane	1.90
DCC in 50% dimethylformamide in methanol	0.45
DCC in 100% dimethylformamide	1.10
Water-soluble carbodiimide, pH 5.0	0.13

When RACS containing immobilized lactase was regenerated, fresh lactase could be immobilized at a concentration of *ca.* 0.35 mg per milliliter of packed gel.

In previous communications[2,3,5,6] we have reported the preparation of immobilized forms of papain, lactase and mitochondrial ATPase which were highly active and possessed the same kinetic properties as did the soluble forms of the enzymes. Immobilization of these enzymes to RACS gave gels with 0.25 mg of papain, 0.05 mg of mitochondrial ATPase and 0.4 mg of lipase per milliliter of

TABLE II

EFFECT OF REGENERATION ON THE ABILITY OF RACS TO REACT WITH L-VALINE METHYL ESTER OR LACTASE

Cycle No.	*μmol L-[^{14}C]valine methyl ester per ml packed gel*	*Lactase bound (mg)*
0	2.2	0.40
1	1.9	0.33
2	1.8	0.32
3	2.2	0.34
4	2.2	0.37
5	2.2	0.33

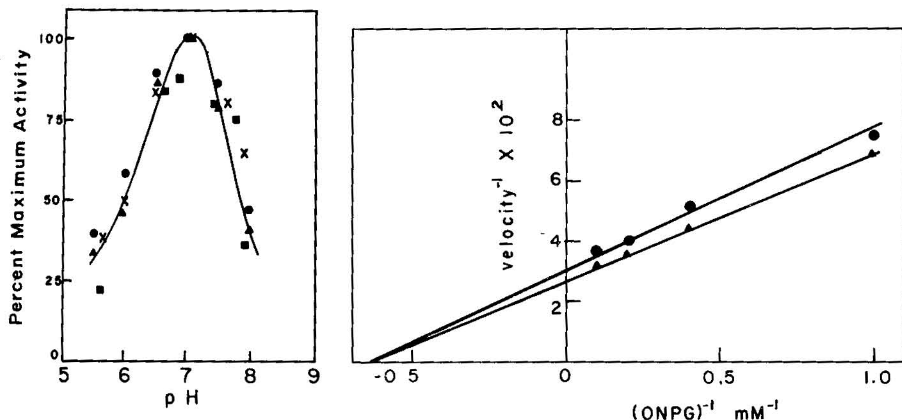


Fig. 3. Enzymatic parameters of lactase (*Saccharomyces lactis*) immobilized to RACS using ONPG as the substrate. ●—●, soluble lactase; ■—■, lactase immobilized to RACS recycled once; ▲—▲, enzyme immobilized to RACS recycled three times; ×—×, enzyme immobilized to RACS recycled five times. A, pH optimum; B, Lineweaver–Burk plot.

packed gel. Coupling of organic amines, in this case proteins, to N-hydroxysuccinimide-activated resin in aqueous solution is a competitive reaction between the amine acid and water. Thus amounts of protein coupled to the resin were a function not only of pH[1], but also of their molar concentrations. Higher levels should be obtained by using more concentrated solutions of proteins. Mitochondrial ATPase immobilized to RACS retained its ability to be activated by bicarbonate, as reported previously for the same enzyme immobilized to succinylaminoethyl Sepharose 4B[5]. Thus it appears that RACS has a broad applicability as a resin for the immobilization of proteins with minimal modification of biological activity.

The concept of RACS originated from a need for a reuseable affinity support principally for the preparation of large-scale immobilized enzyme processing applications. In such an endeavor, the cost of replacing the support when immobilized enzyme is denatured is often limiting. The synthesis of RACS using Sepharose 4B should be feasible using other supports, including controlled pore glass beads, Sephadex, polyacrylamide or any other support on to which a free aromatic amino group can be attached. This compound can be diazotized and coupled with a phenolic compound or aromatic amine. Moreover, regenerable resins with reactive groups other than the carboxyl group should be readily synthesized by judicious choice of the phenolic or aromatic amine to generate the azo linkage to the support. Finally, a single batch of RACS could be recycled to attach proteins of markedly different activity or function, depending on the immediate research demands.

ACKNOWLEDGEMENTS

Published as paper No. 5517, Journal Series, Nebraska Agricultural Experiment Station. The work was conducted under Project No. 15–19.

REFERENCES

1 P. Cuatrecasas and I. Parikh, *Biochemistry*, 11 (1972) 2292.
2 A. Kilara, K. M. Shahani and F. W. Wagner, *Lebensm.-Wiss. Technol.*, 10 (1977) 84.
3 A. Kilara, K. M. Shahani and F. W. Wagner, *Biotech. Bioeng.*, 19 (1977) 1703.
4 H. M. Barnhart, L. B. Bullerman, E. M. Ball and F. W. Wagner, *J. Food Sci.*, 41 (1976) 903.
5 S. Schuster and F. W. Wagner, *J. Chromatogr.*, 157 (1978) 396.
6 A. Kilara, K. M. Shahani and F. W. Wagner, *Food Biochem.*, 1 (1977) 261.
7 H. H. Weetall, in R. B. Dunlap (Editor), *Immobilized Biochemicals and Affinity Supports*, Plenum, New York, 1975, p. 191.
8 P. Cuatrecasas, *J. Biol. Chem.*, 245 (1970) 3059.
9 H. Schlenk and J. L. Gellerman, *Anal. Chem.*, 32 (1960) 1412.
10 G. N. Wilkinson, *Biochem. J.*, 80 (1961) 324.
11 R. M. Perry, Jr., R. C. Chandan and K. M. Shahani, *J. Dairy Sci.*, 49 (1966) 356.

Journal of Chromatography, 176 (1979) 19–24
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,878

ELECTRON CAPTURE GAS CHROMATOGRAPHY WITH SPLITLESS INJECTION ON ISOTHERMALLY OPERATED WIDE-BORE GLASS CAPILLARY COLUMNS

HARALD BRÖTELL*

Astra Pharmaceuticals AB, S-151 85 Södertälje (Sweden)

NILS-OTTO AHNFELT

Department of Analytical Pharmaceutical Chemistry, University of Uppsala, Box 574, S-751 23 Uppsala (Sweden)

and

HANS EHRSSON and STAFFAN EKSBORG

Karolinska Pharmacy, Fack, S-104 01 Stockholm (Sweden)

(First received February 9th, 1979; revised manuscript received March 22nd, 1979)

SUMMARY

The injection of large volumes (up to 50 μl) of diluted samples into a wide-bore isothermally operated glass capillary column has been studied.

A series of *n*-alkanes (C_{11}–C_{16}) have been evaluated as sample solvents by measurement of the resolution between the pesticides *p,p'*-DDD and *o,p'*-DDT. Variations in retention time for the solutes injected in different solvents and in different volumes of the same solvent have been measured. The effect on resolution of changing the injector port temperature has been studied at a constant column temperature of 210°.

A double-injection technique utilizing a combination of a high-boiling (C_{15}) and a low-boiling (C_6) *n*-alkane solvent is described.

More than 500 injections with sample volumes of ≥5 μl have been made on a 0.77 mm I.D. SE-30 column without significant deterioration of its performance.

INTRODUCTION

The splitless injection technique allows injection of large volumes of diluted samples on capillary columns[1]. Most work in this area has so far been done with flame ionization detection and temperature programming of the column. The electron capture detector (ECD) is extremely sensitive to temperature changes, and for this reason it was desirable to apply the splitless injection technique to isothermal analysis.

* To whom correspondence should be addressed. Present address: AB KABI, Research Department, Analytical Chemistry, S-112 87 Stockholm, Sweden.

To achieve a high column efficiency and minimum quenching of the ECD the choice of solvent is critical. The splitless isothermal technique has been utilized by Buser in the analysis of polychlorinated dibenzo-*p*-dioxines[2,3] with *n*-tetradecane as solvent at column temperatures between 205° and 225°. Buser used a high injector port temperature (350°) in combination with injection periods of 20 sec, resulting in 90–95% transfer of sample on to the column[2]. In the present study it was possible to keep the split valve closed, giving 100% transfer with an injector port temperature only slightly above that of the column.

The influence of solvent volatility and injection volume on resolution has been studied, using C_{11}–C_{16} *n*-alkanes as solvents.

A double solvent injection technique, comprising initial injection of a high-boiling *n*-alkane followed by the sample in a low-boiling solvent, is described.

Wide-bore capillary columns with helium as carrier gas were chosen. They have a large sample capacity and permit a high volume flow of gas. Hence the construction of "heart-cutting" systems will be facilitated.

EXPERIMENTAL

Apparatus

A Varian 1400 gas chromatograph was fitted with an injector for split/splitless operation (Ultrasep, Turku, Finland). It was maintained at 250° unless otherwise stated.

The SE-30 glass capillary column (10 m × 0.77 mm I.D., film thickness 0.48 μm) was purchased from Ultrasep. It was operated isothermally at 210° throughout this study. Helium was used as carrier gas, with an inlet pressure of 0.2 bar giving a flow-rate of 4.7 ml/min.

The ^{63}Ni ECD of d.c. type was operated at 275°, and 40 ml/min of nitrogen was added through the hydrogen inlet in the detector base. The electrometer setting was either 4×10^{-10} or 8×10^{-10}A.

Chemicals

n-Hexane and *n*-heptane were of analytical grade quality (Merck, Darmstadt, G.F.R.). C_{11}–C_{16} *n*-alkanes (>99% pure) were obtained from Fluka (Buchs, Switzerland) and were used as received. Pesticides were gifts from the Swedish Agricultural Laboratory (Uppsala, Sweden).

A stock solution of the pesticides in *n*-hexane was prepared. Test solutions of *ca.* 1.2×10^{-8} *M* of each pesticide were prepared by evaporation of an appropriate volume of the stock solution and redissolving in the *n*-alkane to be used.

Injections

Usually 5 μl were injected into the gas chromatograph, corresponding to *ca.* 20 pg of each pesticide.

Splitless injection was performed without septum purge flow and without flushing of the injector port throughout this study. The septum purge and split line valves were kept closed in order to assure a constant flow of carrier gas through the column.

The double solvent injection technique was performed by first rapidly intro-

ducing a high-boiling n-alkane (*ca.* 1 sec). About 5 sec later the sample dissolved in n-hexane was injected with a separate syringe (5 μl in 3–4 sec).

RESULTS AND DISCUSSION

The isothermal splitless injection technique gives retention time variation for the solutes with different solvents and injection volumes. Therefore, calculation of plate numbers is misleading, and column performance was evaluated by measuring the resolution of the two partly overlapping peaks (p,p'-DDD and o,p'-DDT) according to the formula:

$$R_S = \frac{2\Delta V_R}{w_1 + w_2}$$

where ΔV_R is the distance between peak maxima and w_1 and w_2 are the peak widths at base.

The injector purge delay time is a critical factor when splitless injection is used for quantitative work. Alkane solvents in conjunction with flame ionization detection give broad solvent peaks, and therefore purge delay times of less than *ca.* 1 min must be used. This usually means that solutes of high molecular weight are partially lost through the split line[4]. With the combination of electron capture detection and alkane solvents a closed split valve can be used; thus solute losses are avoided. Furthermore, retention times can be measured accurately because the flow of the carrier gas is constant throughout the analysis.

Choice of solvent

Condensation of the solvent in the first part of the column is of crucial importance when the splitless injection technique is used. Thus, the choice of solvent will depend on the column temperature. Table I shows that the solvent should not be more volatile than n-tetradecane at a column temperature of 210°, which is in agreement with the recommendation given by Buser[2]. The use of C_{10} and lower n-alkanes led to severe peak distortion and resolution could not be calculated. n-Alkanes gave a very low ECD response, and large amounts could be injected without excessively broad solvent fronts appearing although the split valve was kept closed.

TABLE I

RESOLUTION OF p,p'-DDD AND o,p'-DDT INJECTED IN 5.0 μl OF DIFFERENT SOLVENTS AT A COLUMN TEMPERATURE OF 210°

Injector port temperature, 250°.

n-Alkane solvent	*Boiling point (°C)*	*R_s (mean values)*	*Relative standard deviation (%) ($n = 10$)*
C_{11}	196	0.88	4.87
C_{12}	216	1.05	5.38
C_{13}	235	1.16	2.47
C_{14}	253	1.22	1.55
C_{15}	271	1.21	4.31
C_{16}	287	1.21	4.93

Solvent volume

The influence of solvent volume on resolution is shown in Fig. 1. Optimum resolution is obtained with injected volumes of 2–4 μl. Too a small solvent volume leads to a sharp decrease in resolution, showing that the solvent in itself is indispensable (*cf.* ref. 5).

The possibility of injecting large volumes of solvent on the wide-bore capillary is also illustrated in Fig. 1. No drastic decrease in resolution was observed even with an injection volume of 100 μl. However, the broad solvent front and impurities in the solvent prevented accurate calculation of the resolution in this case.

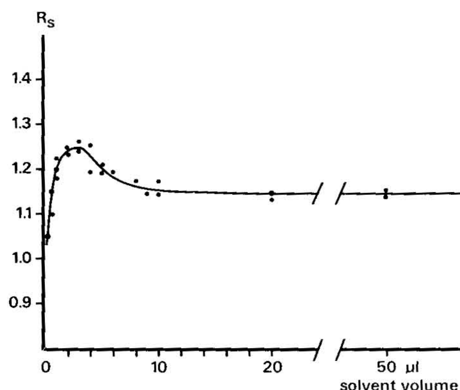


Fig. 1. Resolution of *p,p'*-DDD and *o,p'*-DDT as a function of solvent (*n*-pentadecane) volume.

Droplet and lens formation was clearly observed in the first part of the column with injection volumes of ≥5 μl of *n*-tetradecane or *n*-pentadecane. Nevertheless, the column did not significantly deteriorate during this study, which comprised more than 500 injections.

Injector port temperature

For heat-sensitive samples it is important to use a low injector port temperature. Therefore, the influence of injector port temperature on resolution was studied. The injector port temperature need be only slightly above that of the column (Fig. 2), the split valve being closed to ensure a quantitative transfer of the solutes into the column.

Retention time variation

The retention time for a solute is a function of both solvent volatility (Fig. 3) and solvent volume (Fig. 4). The increase in condensation with the heavier alkane solvents is accompanied by an increase in resolution (Table I), indicating that the condensed amount of solvent with C_{11}–C_{13} is too small to take full advantage of the solvent effect. Fig. 3 indicates a parallel shift to longer retention times for all solutes when going from C_{13} to C_{16} as solvent.

The increase in retention time with larger injection volumes is shown in Fig. 4, which indicates a linear relationship between 10 and 100 μl. This effect must be kept in mind, especially when utilizing the technique for qualitative purposes.

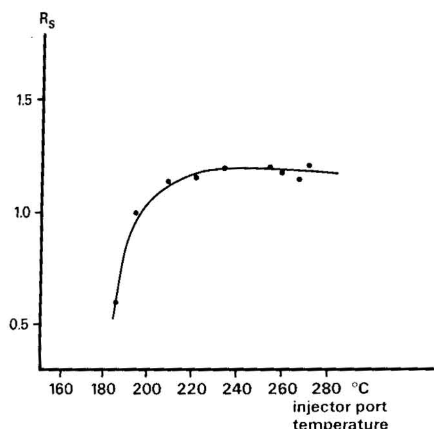


Fig. 2. Resolution as a function of injector port temperature. *p,p'*-DDD and *o,p'*-DDT were injected in 5.0 μl of *n*-pentadecane. Column temperature: 210°.

Double solvent injection

The usefulness of the present technique is hampered by the very limited choice of sample solvent. This drawback was overcome by injection of a suitable high-boiling alkane as a "barrier", followed by injection of the sample dissolved in a volatile solvent, which if it were injected alone would lead to poor resolution (Fig. 5A). The resolution of *p,p'*-DDD and *o,p'*-DDT obtained with this double solvent injection (Fig. 5B) is comparable with that obtained with a split injection (Fig. 5C). The influence of the amount of C_{15} *n*-alkane injected prior to 5 μl of *n*-hexane is illustrated in Fig. 6. The volume of the C_{15} *n*-alkane "barrier" should not be less than 5 μl if the resolution is to be maintained.

The resolution decreases with increasing time delay between injections (Fig. 7).

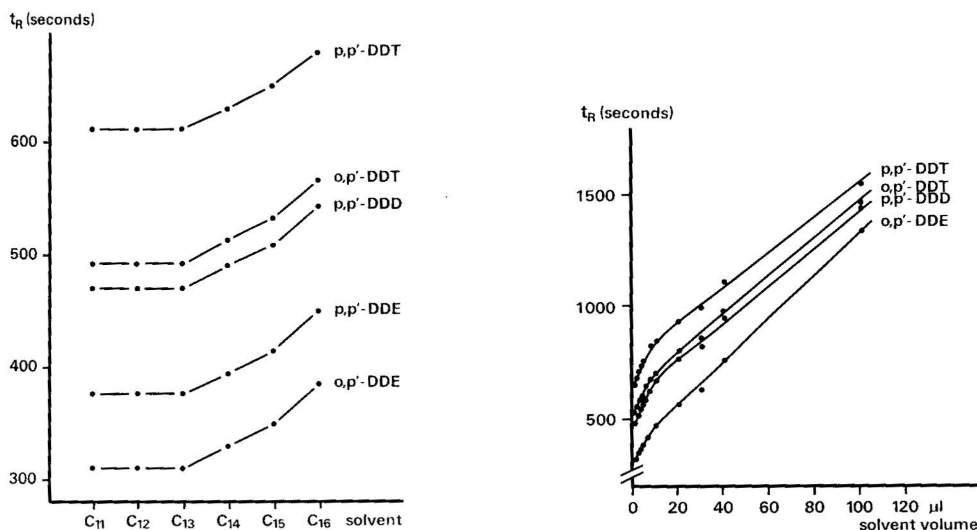


Fig. 3. Variation in retention time for a pesticide mixture injected in different solvents at a column temperature of 210°.

Fig. 4. Variation in retention time for a pesticide mixture injected in different volumes of *n*-pentadecane at a column temperature of 210°.

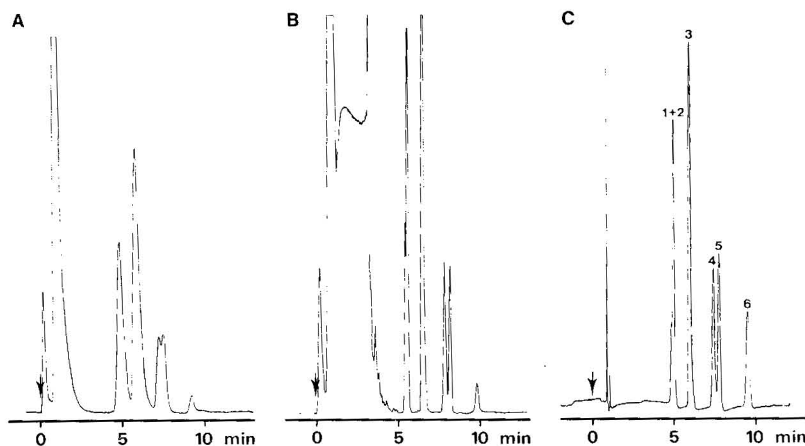


Fig. 5. Chromatograms of the pesticide mixture dissolved in *n*-hexane. A, Splitless injection of 5.0 μl of *n*-hexane solution. B, Double solvent injection. 5.0 μl of *n*-pentadecane injected prior to 5.0 μl of the *n*-hexane pesticide solution. Time between injections was *ca.* 5 sec. C, Split injection of 0.2 μl with a split ratio of 1:15. 1, *p,p'*-DDMU; 2, *o,p'*-DDE; 3, *p,p'*-DDE; 4, *p,p'*-DDD; 5, *o,p'*-DDT; 6, *p,p'*-DDT.

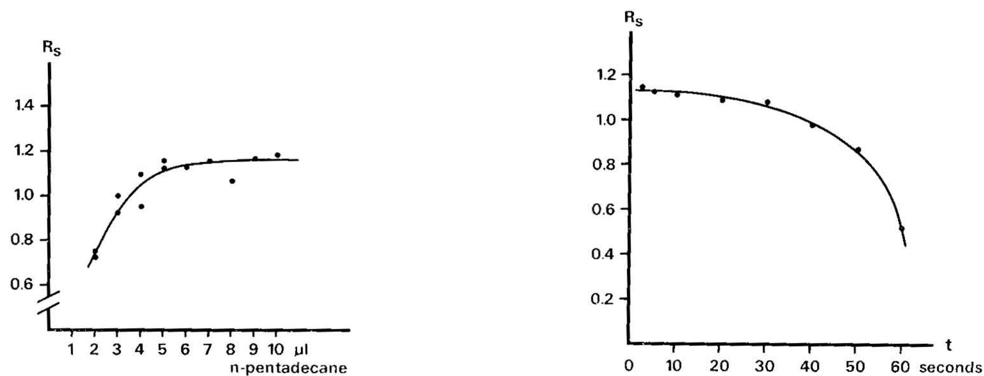


Fig. 6. Resolution as a function of the amount of *n*-pentadecane injected prior to 5.0 μl of *n*-hexane containing *p,p'*-DDD and *o,p'*-DDT.

Fig. 7. Resolution as a function of time elapsed between injection of 5 μl of *n*-pentadecane prior to the solutes in 5 μl of *n*-hexane.

To take advantage of the double solvent injection technique the delay should not exceed *ca.* 30 sec. A delay of 3–4 sec is easily achieved after some practice.

ACKNOWLEDGEMENT

The authors thank Prof. G. Schill and Prof. J. Vessman for their comments on the manuscript.

REFERENCES

1 K. Grob and K. Grob, Jr., *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 1 (1978) 57.
2 H. R. Buser, *Anal. Chem.*, 48 (1976) 1553.
3 H. R. Buser, *Anal. Chem.*, 49 (1977) 918.
4 F. I. Yang, A. C. Brown, III and S. P. Cram, *J. Chromatogr.*, 158 (1978) 91.
5 K. Grob and K. Grob, Jr., *J. Chromatogr.*, 94 (1974) 53.

Journal of Chromatography, 176 (1979) 25–35
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,879

USE OF SPECIFIC RETENTION VOLUMES IN THE EVALUATION OF VARIOUS TYPES OF COLUMNS FOR USE IN THE TRACE DETERMINATION OF ETHYLENE GLYCOL BY GAS CHROMATOGRAPHY

MICHAEL E. KASHTOCK

Food and Drug Administration, Division of Chemistry and Physics, Washington, D.C. 20204 (U.S.A.)

(Received February 13th, 1979)

SUMMARY

Various types of column packings were evaluated for use in the gas chromatographic determination of ppm concentrations of ethylene glycol in aqueous systems. The columns were evaluated by determining specific retention volumes and observing peak shapes for progressively smaller concentrations of ethylene glycol in water injected on to the column. An increase in the specific retention volume, accompanied by significant peak tailing, was taken to indicate that quantitative analysis would not be feasible below a certain concentration range, because of chemisorptive effects. The most desirable column for quantitative analysis at trace levels (10 ppm or more injected on to the column) was selected on the basis of comparative evaluation. Acid-washed and silane-treated diatomaceous supports coated with Carbowax 20M, uncoated porous polymers (Chromosorb 101 and 102), and Chromosorb 102 coated with Carbowax 20M were considered unsatisfactory. Super-Pak 20M coated with Carbowax 20M was marginally successful, while Chromosorb 101 coated with Carbowax 20M was clearly the column packing of choice. These results are discussed in terms of column support adsorption effects.

INTRODUCTION

The determination of trace levels of polar organic compounds by conventional gas–liquid chromatography (GLC) on diatomaceous supports is often difficult because of adsorption effects resulting in increasing retention volumes accompanied by peak tailing and irreproducible responses. These effects are usually caused by hydroxyl groups on the surface of the support. This problem is not confined to low loads of stationary phase where incomplete surface coverage might be implicated, but can exist with completely coated supports[1–3]. The use of silane-treated supports may improve performance in this respect. However, adsorption problems have been reported that could be attributed to poor silanization of commercially available supports of this type[4] or possible adsorption at the silyl group[5].

An alternative choice for the gas chromatographic stationary phase is to use porous polymers produced from divinylbenzene and either styrene or ethylvinyl-

benzene. Presumably no hydroxyl groups are present in these materials to strongly adsorb polar compounds. However, unreacted vinyl groups[6], residual transition metals[7,8] and unspecified causes[9,10] have been implicated in adsorption of compounds such as amines[6,8], volatile free fatty acids[6,9] and alcohols[6,10] at low solute concentrations. Some workers[6,8,11–13] have improved performance in trace analysis by coating porous polymers with common liquid phases.

In our work to determine ethylene glycol (EG) migrating at trace levels from poly(ethylene terephthalate) bottles into aqueous food-simulating solvents, we observed problems similar to those often encountered in the chromatography of polar compounds. To select an appropriate GLC column, we evaluated various types of columns by determining specific retention volumes and observing peak shapes for progressively smaller concentrations of EG in water injected on to the columns. This comparative evaluation resulted in the selection of a column which is being used for EG analysis with a minimum of adsorption-related problems.

EXPERIMENTAL

Apparatus and general procedure

The gas chromatograph used for this study was a Hewlett-Packard 5840A equipped with a flame ionization detector. Nitrogen carrier gas was passed through an oxygen trap installed in the carrier gas line to the instrument. The injection port was maintained at 195° and the detector at 225°.

The various column packings were packed with gentle tapping and vacuum into 6 ft. × 2 mm I.D. glass coils to allow "on-column" injection of the sample. All columns were evaluated at 170° by determining specific retention volumes (as defined in Results and discussion) and observing peak shapes for EG as a function of the quantity of EG injected. Triplicate 5 μl injections were made of each solution of EG in distilled water, in the concentration range of 8 ppm to 2 parts per thousand (v/v). The EG retention times for each concentration were averaged to calculate retention volumes.

Coated diatomaceous supports

An approximate 10% Carbowax 20M on 80–100 mesh Chromosorb W AW packing was prepared by standard solution coating from chloroform. The exact loading was determined by Soxhlet extraction with chloroform. Residual oxygen was purged from the column at room temperature using nitrogen flow for 30 min. After conditioning with flow for 2 h at 270°, the column was cooled to 170° and evaluated. The same column was then further conditioned at 270° for an additional 16 h and re-evaluated. The column was finally conditioned at 270° for 68 h more and re-evaluated. Immediately after the final evaluation, the amount of Carbowax 20M remaining on the support was determined by Soxhlet extraction of the packing removed from the column.

A similar procedure was followed for a column packed with 10% Carbowax 20M on Super-Pak 20M (mesh size not specified by manufacturer) and a column packed with 20% Carbowax 20M on 60–80 mesh Chromosorb WHP.

Carrier gas flow-rates for all evaluations of the coated diatomaceous supports were 28–30 ml/min. Column head pressures were *ca.* 30, 19 and 49 p.s.i.g. for the

Chromosorb W AW, Chromosorb W HP and Super-Pak 20M support groups, respectively.

Porous polymers and coated porous polymers

Chromosorb 101 and 102 (80–100 mesh) columns were purged with nitrogen at ambient temperature for 30 min. The columns were conditioned with flow at 200° for 4 h, after which they were evaluated. After the initial evaluation each column was brought to ambient temperature under continued nitrogen flow. To assess the effect of residual oxygen during conditioning the following routine was performed. The carrier gas line of the gas chromatograph was disconnected from the nitrogen source and connected to a source of air of breathing quality. The flow-rate was set at 50 ml/min and continued for 30 min at ambient temperature. The carrier gas line was reconnected to the nitrogen source, the flow was initiated, and the column oven temperature was immediately raised to 200° and held for 4 h. After this procedure, the columns were again evaluated . Chromosorbs 101 and 102 from the same lots as the above were each coated with 3 and 6% Carbowax 20M by solution coating from chloroform. Each column was purged with nitrogen at ambient temperature for 30 min, conditioned with flow for 4 h at 200° and evaluated.

Evaluations of the porous polymers were performed at carrier gas flow-rates of 22.0 and 24.0 ml/min for the Chromosorb 101 and 102 groups, respectively. This was done to avoid the variation within each group of retention volumes with the carrier gas flow-rate, which has been reported to occur for porous polymers[14,15]. Column head pressures were *ca.* 27 and 44 p.s.i.g. for uncoated Chromosorbs 101 and 102, 39 p.s.i.g. for both 3 and 6% Carbowax 20M on Chromosorb 101, and 41 p.s.i.g. for both 3 and 6% Carbowax 20M on Chromosorb 102. Two columns were packed from each lot of prepared packing material. Since the results were in general agreement for all sets of duplicate columns, results for only one column from each set are presented.

RESULTS AND DISCUSSION

Coated diatomaceous supports

Fig. 1 shows the "apparent" specific retention volumes for EG after the various conditioning periods for the Carbowax 20M on Chromosorb W AW column. We define "apparent" specific retention volume as follows:

$$V_g^{T'} = V_n / W_s'$$

where $V_g^{T'}$ is the "apparent" specific retention volume (at 170°), V_n is the net retention volume corrected for dead volume and pressure drop[16], and W_s' is weight of stationary phase in the unconditioned packing. Since *ca.* 50% of the initial load on the support was lost during the total conditioning and evaluation procedure, the apparent values are not true specific retention volumes as required for the determination of thermodynamic parameters. They deviate increasingly from the true values for the more highly conditioned columns. The slopes of the curves for the highly conditioned columns are also less than what would be observed if actual specific retention volumes were used. However, any dependence of retention volume on the concentration of EG injected is still evident. Retention volumes are expressed relative to the unconditioned

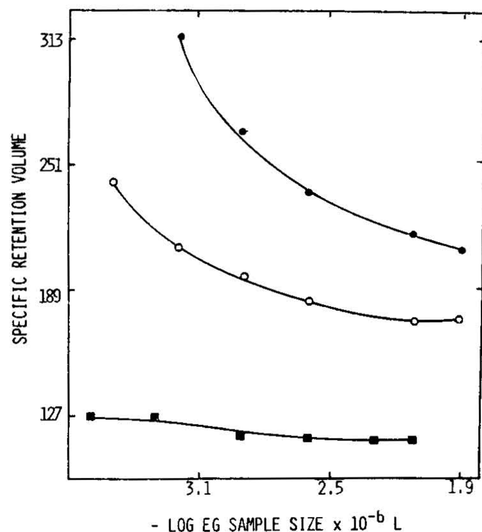


Fig. 1. "Apparent" specific retention volume at 170° (per gram of Carbowax 20M present in the unconditioned packing) *vs.* EG sample size, for a column of 10% Carbowax 20M on Chromosorb W AW conditioned at 270° for ● 2 h, ○ 18 h and ■ 96 h.

packing to allow for the evaluation of stationary phase bleed, for a single column through the progressive conditioning process. This would be indicated by decreasing apparent specific retention volume values during the cumulative conditioning process.

A concentration dependence of the apparent specific retention volume, such that it increases as smaller amounts of solute are injected, usually indicates solute adsorption at active sites on the support surface. A progressive bleed of stationary phase accompanied by a progressive lessening of support adsorption of EG is indicated by Fig. 1. The latter observation agrees with findings of Aue *et al.*[17], who observed a similar deactivation of the surface of Chromosorb W by Carbowax 20M when such high-temperature conditioning was employed.

However, EG exhibited severe tailing (Fig. 2) with poor response for the initial two evaluations. The dependence of retention volume on quantity injected lessened considerably for the evaluation of the column conditioned for 96 h. However, double peaking due to ghosting was observed, as previously reported in adsorption-plagued analyses[18,19]. Thus, after 96 h of cumulative conditioning at 270° and with 4.6% stationary phase still remaining. Chromosorb W AW was not a satisfactory support for the trace analysis of EG.

Apparent specific retention volumes for EG after the various conditioning periods for the Carbowax 20M on Chromosorb W HP column are shown in Fig. 3. This support also lost *ca.* 50% of its original stationary phase load during the cumulative conditioning process, accounting for the progressive decrease in apparent specific retention volume. Support adsorption effects are clearly evident for each evaluation. However, the effects present during the initial evaluation are considerably less than those observed for the Chromosorb W AW support and are only slightly lessened during the cumulative conditioning process.

These observations indicate that the silanized diatomaceous support offers a

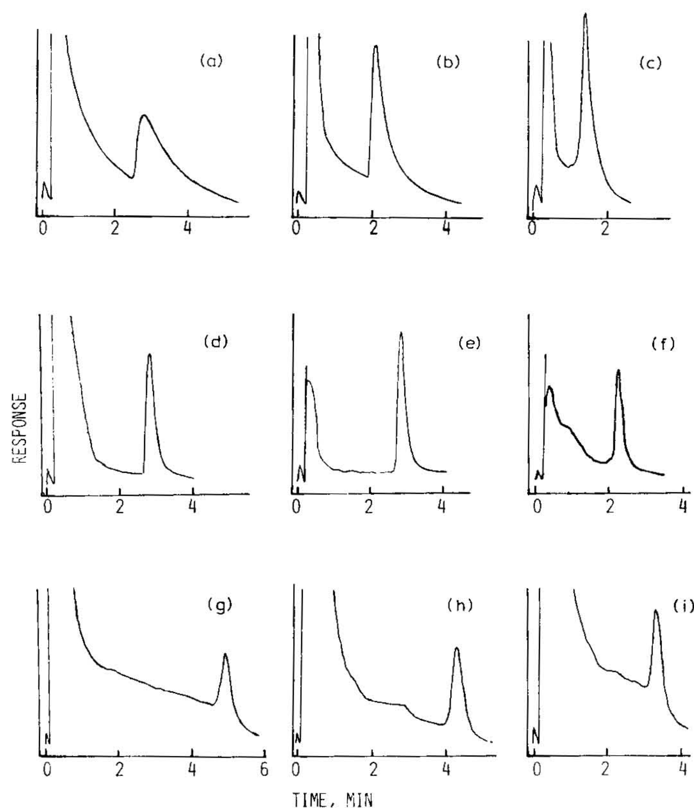


Fig. 2. EG peak shapes obtained at 170° for: (a)–(c), 10% Carbowax 20M on Chromosorb W AW; (d)–(f), 20% Carbowax 20M on Chromosorb W HP; (g)–(i), 10% Carbowax 20M on Super-Pak 20M. Conditioning: (a), (d) and (g), 2 h; (b), (e) and (h), 18 h; (c), (f) and (i), 96 h.

less adsorptive surface than its acid-washed counterpart, as would be expected. However, the potential for achieving further support deactivation through conditioning, when a relatively polar stationary phase is employed, may be discouraged by the hydrophobic nature of the surface of the silanized support. This is evidenced by the observation that after 96 h of cumulative conditioning, the acid-washed support exhibited less support activity than the silanized support. Tailing of EG peaks observed for this column is shown in Fig. 2. Because of persistent adsorption effects, we considered this support unsatisfactory for the chromatography of small amounts of EG. Recognizing, however, that commercially available supports of this type may exhibit widely varying degrees of support activity, it is not unlikely that more carefully silanized supports of this type would exhibit improved performance[4].

The evaluation of the column of Carbowax 20M on Super-Pak 20M is shown in Fig. 4. Once again a *ca.* 50% stationary phase bleed occurred during the cumulative conditioning process, accounting for the progressive decrease in the apparent specific retention volumes. However, this support exhibited virtually no dependence of apparent specific retention volume on sample size during the initial evaluation. Support effects evidenced by changing apparent specific retention volumes were observed

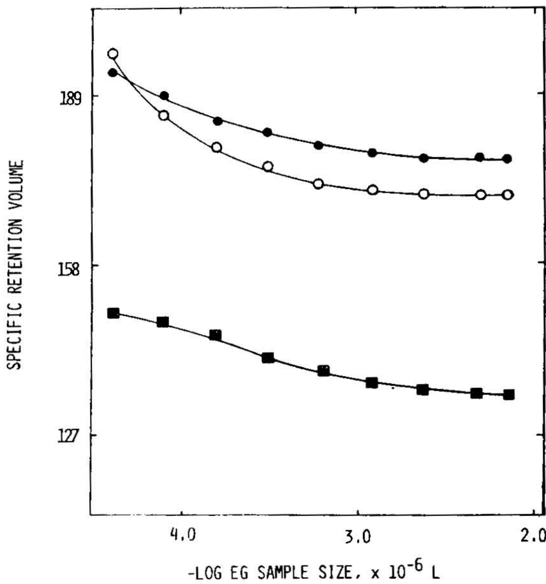


Fig. 3. "Apparent" specific retention volume at 170° (per gram of Carbowax 20M present in the unconditioned packing) *vs.* EG sample size, for a column of 20% Carbowax 20M on Chromosorb W HP conditioned at 270° for ● 2 h, ○ 18 h and ■ 96 h.

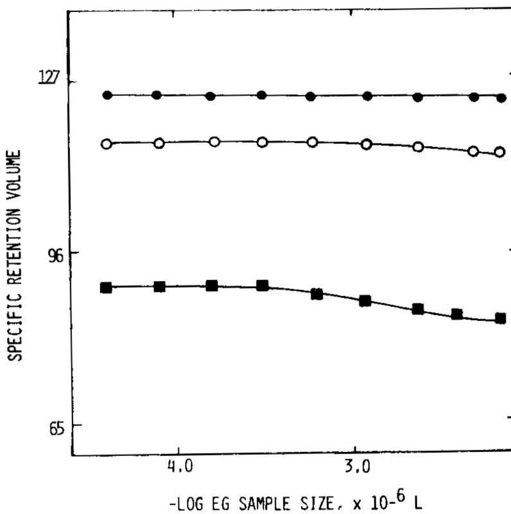


Fig. 4. "Apparent" specific retention volume at 170° (per gram of Carbowax 20M present in the unconditioned packing) *vs.* EG sample size, for a column of 10% Carbowax 20M on Super-Pak 20M conditioned at 270° for ● 2 h, ○ 18 h and ■ 96 h.

for the later evaluations. Excessive high-temperature conditioning is evidently ruinous to the support, as indicated by the manufacturer.

Super-Pak 20M is a specially prepared, deactivated diatomaceous support based on the work of Aue *et al.*[17]. The progressive deactivation of Chromosorb W AW

which we observed (Fig. 1) is commercially employed to produce Super-Pak 20M, with the result that this support as supplied is clearly the most suitable diatomaceous type of support for this analysis.

EG peak shapes obtained for this column are nearly symmetrical for the initial evaluation, as shown in Fig. 2. However, curves for response *vs.* amount of EG injected for the 2 h conditioned column showed considerable irreproducibility of integration values for EG peaks, indicating that some support adsorption may still be occurring. For this reason it was decided to evaluate non-diatomaceous supports in an attempt to ascertain what would be an optimal column for this analysis.

Porous polymers and coated porous polymers

Figs. 5 and 6 show specific retention volumes (at 170°) expressed per gram of Chromosorb 101 and 102, respectively, for these two uncoated porous polymers conditioned with and without residual oxygen present in the column. For both of the uncoated porous polymers which have been purged of residual oxygen prior to conditioning, trace analysis of EG is complicated by active-site adsorption effects. Such effects are attributed to the presence of unreacted vinyl groups and residual transition metals in the porous polymers, as previously mentioned.

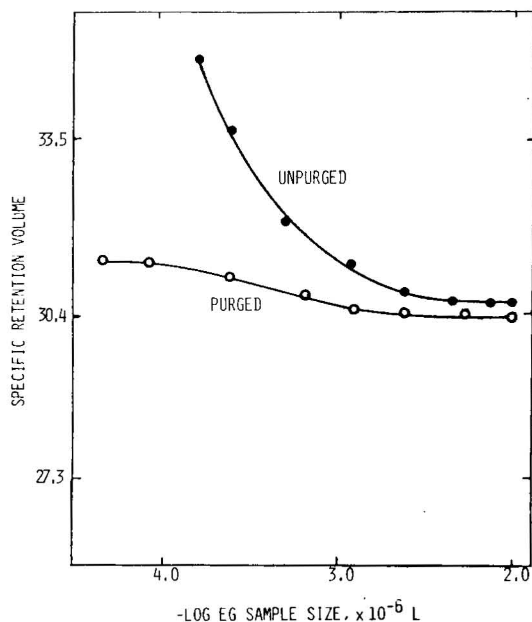


Fig. 5. Specific retention volume at 170° (per gram of Chromosorb 101) *vs.* EG sample size, for uncoated Chromosorb 101 conditioned with (●) and without (○) residual air present in the packed column.

It is especially detrimental to the performance of these porous polymers if they are not purged of residual oxygen prior to high-temperature conditioning. Neumann and Morales T have associated the formation of conjugated carbonyl compounds with a reduction of residual vinyl content in Chromosorb 102 heated at 200°

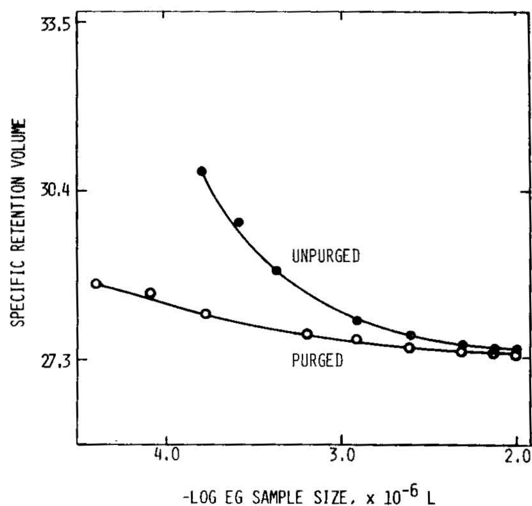


Fig. 6. Specific retention volume at 170° (per gram of Chromosorb 102) *vs.* EG sample size, for uncoated Chromosorb 102 conditioned with (●) and without (○) residual air present in the packed column.

in an oxygen-containing nitrogen flow[20]. The formation of carbonyls on the surface of these porous polymers presents the potential for an enhanced interaction with compounds possessing hydroxyl groups such as EG. Peak shapes for EG obtained for these columns are shown in Fig. 7A–D. As expected, increased tailing is observed for the columns which were not purged of residual oxygen prior to conditioning at the elevated temperature.

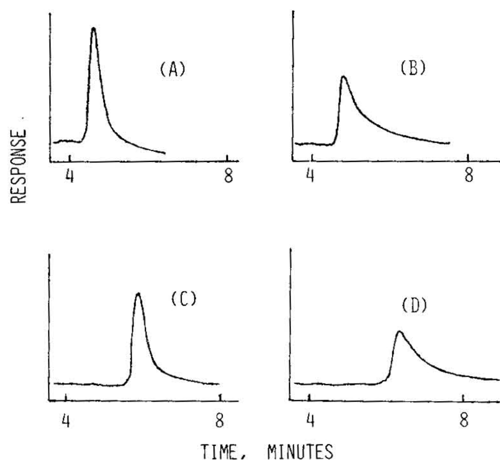


Fig. 7. EG peak shapes obtained for uncoated Chromosorb 101 and 102 columns. Chromatograms A and B represent Chromosorb 101 columns conditioned without and with residual air present in the packed column. Chromatograms C and D are the equivalent pair for Chromosorb 102. The EG sample size in each case is *ca.* 5 times larger than the minimum amount detectable.

The effect of coating Chromosorb 101 with a liquid phase is illustrated in Fig. 8. With 3 and 6% loads of Carbowax 20M, there is no dependence of the specific retention volume (per gram of Chromosorb) on the amount of EG injected. Furthermore, peak shapes are nearly symmetrical for small amounts of EG injected, as shown in Fig. 9A–B, and EG peak integration values are reproducible. However, for 3 and 6% loads of Carbowax 20M on Chromosorb 102, a dependence of the specific retention volume on the amount of EG injected still exists, although this dependence is less for the 6% column as shown in Fig. 10. Peak shapes shown in Fig. 9C–D indicate tailing for small amounts of EG injected.

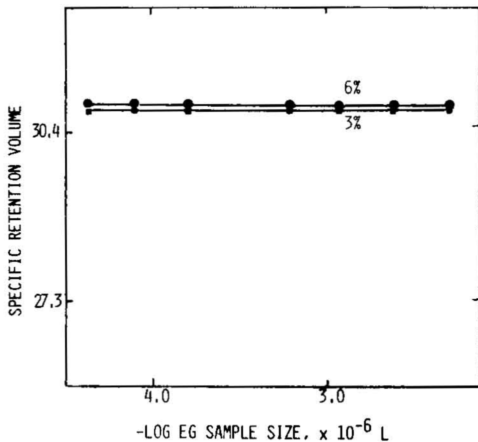


Fig. 8. Specific retention volume at 170° (per gram of Chromosorb 101) *vs.* EG sample size for coated Chromosorb 101, where the liquid phase is (●) 6% Carbowax 20M and (■) 3% Carbowax 20M.

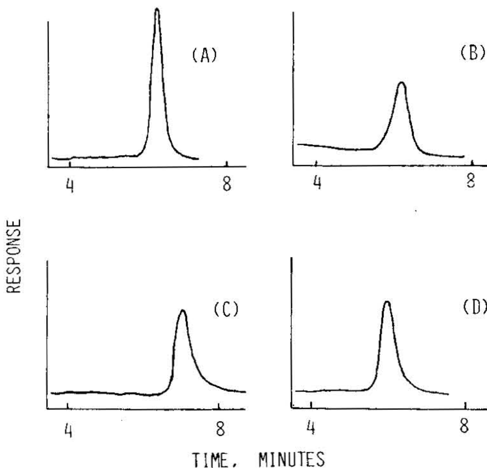


Fig. 9. EG peak shapes obtained at 170° for coated Chromosorb 101 and 102 columns. Chromatograms A and B represent coated Chromosorb 101 where the liquid phase is 3% and 6% Carbowax 20M. Chromatograms C and D are the equivalent pair for coated Chromosorb 102. The EG sample size in each case is *ca.* 5 times larger than the minimum amount detectable.

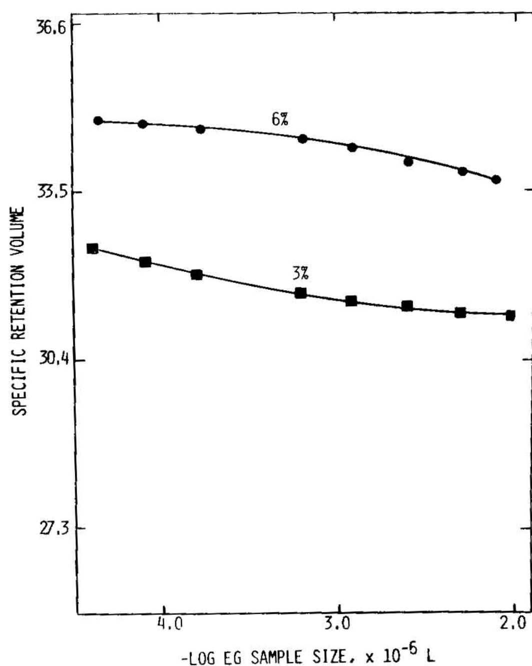


Fig. 10. Specific retention volume at 170° (per gram of Chromosorb 102) *vs.* EG sample size for coated Chromosorb 102, where the liquid phase is (●) 6% Carbowax 20M and (■) 3% Carbowax 20M.

When examining the effect of 3 and 6% Carbowax 20M on the specific retention volumes per gram of Chromosorb 101 and 102, it is apparent that the presence of a liquid phase has a much greater effect relative to the uncoated porous polymer for Chromosorb 102. Although these two materials are chemically similar, they are markedly dissimilar with respect to surface area and average pore diameter, with corresponding values of 40 m^2/g and 3000–4000 Å and 300–400 m^2/g and 85 Å for Chromosorbs 101 and 102, respectively[21].

The microporous structure of Chromosorb 102 probably leads to a pore-filling effect upon coating with Carbowax 20M such that bulk liquid phase deposited in the micropores contributes to a substantial increase in the specific retention volume of EG relative to uncoated Chromosorb 102. While bulk liquid phase is pooled in micropores upon coating, portions of the support surface remain relatively uncoated, contributing to the observed adsorption effects when coated Chromosorb 102 is evaluated. This represents a combination gas–solid and gas–liquid chromatography, as reported for coated Porapak Q[8], with significant support adsorption effects present even at a 6% liquid phase load.

For coated Chromosorb 101, the microporous structure of this support probably favors the deposition of a thin layer of Carbowax 20M which possesses non-bulk properties, since the specific retention volumes relative to the uncoated support increased only slightly. The more complete surface coverage attained in the absence of pooling of the liquid on this support accounts for the superior suppression of adsorp-

tion effects on coated Chromosorb 101. Specific retention volumes relative to uncoated Chromosorb 101 indicate that a somewhat modified gas–solid chromatography occurs for the coated Chromosorb 101 columns.

The suppression of support adsorption effects obtained for the coated Chromosorb columns is attributed to a physical blocking of active sites on the surface of the porous polymer, as discussed by Hertl and Neumann[6]. They suggested that treating porous polymers containing residual vinyl groups with HF would be a more desirable method for deactivating these active sites. Merely coating the surface of the polymer with a liquid phase presents the possibility for loss of the liquid phase through bleeding and an eventual availability of active sites on the support surface.

We found the columns of 3 and 6% Carbowax 20M on Chromosorb 101 to be suitable for determination of low concentrations of EG as long as the above limitation is recognized. There does seem to be a sudden onset of irreproducible integration values for EG peaks if a column has been in continuous use at 170° for a month or so. This may be due to a gradual bleed of liquid phase resulting in a subsequent availability of support active sites as mentioned earlier. We are now reducing the temperature of the column oven to 50° when analyses are not being performed, in the hope of extending column life as recommended by Ives and Giuffrida[22].

REFERENCES

1 J. C. Giddings, *Anal. Chem.*, 35 (1963) 440.
2 P. Urone, Y. Takahashi and G. H. Kennedy, *Anal. Chem.*, 40 (1968) 1130.
3 C. R. Hastings and W. A. Aue, *J. Chromatogr.*, 89 (1974) 369.
4 R. Roman, C. H. Yates and J. F. Millar, *J. Chromatogr. Sci.*, 15 (1977) 555.
5 J. J. Pesek and J. E. Daniels, *J. Chromatogr. Sci.*, 14 (1976) 288.
6 W. Hertl and M. G. Neumann, *J. Chromatogr.*, 60 (1971) 319.
7 H. L. Gearhart and M. F. Burke, *J. Chromatogr. Sci.*, 15 (1977) 1.
8 J. R. Lindsay Smith and D. J. Waddington, *Anal. Chem.*, 40 (1968) 522.
9 R. G. Ackman, *J. Chromatogr. Sci.*, 10 (1972) 560.
10 M. Gassiot-Matas and B. Monrabal-Bas, *Chromatographia*, 3 (1970) 547.
11 B. O. Jansson, K. C. Hallgren and G. Widmark, *J. Chromatogr. Sci.*, 8 (1970) 398.
12 O. L. Hollis, *Anal. Chem.*, 38 (1966) 309.
13 H. D. Spitz and J. Weinberger, *J. Pharm. Sci.*, 60 (1971) 271.
14 N. R. Rakshieva, J. Novák, S. Wičar and J. Janák, *J. Chromatogr.*, 91 (1974) 51.
15 N. R. Rakshieva, S. Wičar, J. Novák and J. Janák, *J. Chromatogr.*, 91 (1974) 59.
16 A. B. Littlewood, *Gas Chromatography*, Academic Press, New York, 2nd ed., 1970, Ch. 6.
17 W. A. Aue, C. R. Hastings and S. Kapila, *J. Chromatogr.*, 77 (1973) 299.
18 C. Van Eenaeme, J. M. Bienfait, O. Lambot and A. Pondant, *J. Chromatogr. Sci.*, 12 (1974) 398.
19 R. G. Ackman and R. D. Burgher, *Anal. Chem.*, 35 (1963) 647.
20 M. G. Neumann and S. Morales T, *J. Chromatogr.*, 74 (1972) 332.
21 H. L. Gearhart and M. F. Burke, *J. Chromatogr. Sci.*, 11 (1973) 411.
22 N. F. Ives and L. Giuffrida, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 973.

Journal of Chromatography, 176 (1979) 37–41
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,891

SELECTION OF A GAS CHROMATOGRAPHIC MATERIAL FOR USE IN EXPLOSIVES VAPOR PRECONCENTRATION

F. J. CONRAD, T. A. BURROWS and W. DAVID WILLIAMS

Entry-Control Systems Division, Sandia Laboratories, Albuquerque, N.M. 87185 (U.S.A.)

(First received August 14th, 1978; revised manuscript received March 26th, 1979)

SUMMARY

The effectiveness of explosives vapor detectors can be enhanced by the addition of a preconcentrator which collects explosives molecules and releases this concentrated sample to the detector for analysis. Candidate preconcentrator materials for detector applications were evaluated using a gas chromatograph and a commercial explosives vapor detector. The saturation time and release temperature for nine materials were obtained using ethylene glycol dinitrate and 2,4-dinitrotoluene explosives. Based on these data, the best candidate for use in a thin-screen preconcentrator for the above explosives is OV-275.

INTRODUCTION

Commercial explosives detectors are used in a number of security systems to detect explosives carried by individuals. The effectiveness of these detectors is limited, however, by the low concentrations of available explosives vapors. The extent of this problem is illustrated in Table I, which lists the vapor pressures of some common explosives.

TABLE I

EQUILIBRIUM VAPOR PRESSURES OF HIGH EXPLOSIVES AT ROOM TEMPERATURE AND ATMOSPHERIC PRESSURE

Abbreviations: EGDN = ethylene glycol dinitrate; TNT = 2,4,6-trinitrotoluene; DNT = 2,4-dinitrotoluene; PETN = pentaerythritol tetranitrate; RDX = cyclonite; C-4 = principally, cyclonite.

High explosives	*Relative partial concentration*[1–6] *(ppb)*
Dynamite:	
Nitroglycerin	300
EGDN	63,900
TNT	6
DNT	145
PETN (chemically pure)	0.0005
RDX	0.0015
C-4 (91 % RDX)	0.0013

Although progress continues to be made in increasing the sensitivity of explosives vapor detectors, the performance of these detectors could be enhanced by preconcentration of the explosives vapors[7]. Vapor preconcentration can be accomplished by a device which collects a high percentage of small amounts of vapor dispersed throughout a large volume of air. The enriched vapor sample is then delivered to a detector system for analysis. Of particular interest are thin layers of preconcentrator materials which are applied to a screen mesh for use in conjunction with existing explosives vapor detectors.

Several materials were investigated for possible use in explosives vapor preconcentration. Each material was tested to determine vapor collection efficiency and the optimum temperature for vapor release.

EXPERIMENTAL

Columns constructed of 304 stainless-steel tubing were filled with candidate preconcentrator materials on Chromosorb P AW support and used as a first approximation to a thin-layer sample. Each tube measured 5 cm $\times$ 3.2 mm O.D. After being loaded with a candidate material, the ends of each column were closed with a 10-μm stainless-steel screen. The columns were then preconditioned in a flow stream of 95% argon–5% methane. Preconditioning consisted of heating the columns to a temperature of 20° below the maximum operating temperture of the preconcentrator materials or 300°, whichever was lower.

Both saturation time and release temperature were investigated. Saturation time is a measure of the molecular collection efficiency and retention capacity of a material[8]. Materials which exhibit long saturation times are preferred for preconcentration applications. Release temperature is the temperature at which a material releases the captured vapor moleules to the surrounding media. The best release temperature is the lowest temperature above ambient which results in the release of substantial quantities of the captured vapor molecules.

Table II lists the nine candidate preconcentrator materials which were studied. These materials were chosen because they included a wide range of the McReynold's constants[9] and several porous polymer materials which have been used previously as collection media.

Saturation time

The test columns were attached to an explosives-filled glass coil of a P.A. Pella-type generator[10]. Air (Zero Gas, Mathesow) was passedthrough a temperature-controlled coil containing solid explosives material. The explosives vapor effluents from the coil then passed into the test column. Petrogel™, Atlas HV 60% dynamite, was used as the explosive. Since this dynamite contains a high concentration of ethylene glycol dinitrate (EGDN), it will have a high vapor pressure (see Table I). The effluent from the column was monitored continuously with an Ion Track Instruments (ITI) Model 70 explosives vapor detector. Saturation time of the column was recorded as the elapsed time from the start of the effluent flow through the column until the detector produced a continuous alarm.

TABLE II

PROPERTIES OF CANDIDATE PRECONCENTRATION MATERIALS

The activated coconut charcoal (100–200 mesh) was obtained from Coast Engineering Lab. (Gardena, Calif., U.S.A.), the Tenax GC (60–80 mesh) from Applied Science Labs., (State College, Pa., U.S.A.) the Ultrabond 20M (100–200 mesh) from Alltech (Arlington Heights, Ill., U.S.A.); all other materials were obtained from Supelco (Bellefonte, Pa., U.S.A.). Physical form refers to the basic material not the substrate.

Material	*Temp. range (°C)*		*McReynolds constants*[8]					*Physical form*
	Min.	*Max.*	*x′*	*y′*	*z′*	*u′*	*s′*	
3% OV-101	0	250	017	057	045	067	043	viscous liquid
5% OV-275	25	250	629	872	763	1106	849	viscous liquid
5% DEGS-PS	20	200	496	746	590	837	835	viscous liquid
5% Carbowax 20M-TPA	60	2255	321	537	367	573	520	low-melting solid
Tenax GC	–	375	–			–		porous polymer
Porapak Q		250			–	–	–	porous polymer
Activated charcoal		–			–	–	–	solid
5% SP-1200–5% Bentone 34	25	180				–	–	liquid–solid
Ultrabond 20M		250	–	–	–	–	–	solid

Release temperature

A Hewlett-Packard HP-5840A gas chromatograph with an electron capture detector (ECD) was used to determine the release temperature of each of the candidate preconcentrator materials. The test columns were placed into the HP-5840A and subjected to a 95% argon–5% methane gas flow. Separate samples of EGDN and 2,4-dinitrotoluene (2,4-DNT) were dissolved in acetone to a concentration of 98 mg/l and used to test the columns.

The oven of the gas chromatograph was temperature stabilized at 50°. This temperature was selected as the initial oven temperature for collection of the explosives molecules from the explosives–acetone solution since this temperature exceeds the "worst case" temperature that might be encountered in field operation of an explosives vapor detector. Following temperature stabilization, 1 μl of the explosives–acetone solution was injected onto the column, using the solvent plug technique, and the oven temperature-profile program was begun. The oven temperature-profile program contained a 3-min hold at 50° followed by a temperature increase at a rate of 20°/min to 225° and was terminated with a 10-min hold at the maximum temperature. All of the candidate materials were tested using this profile except the 5% SP-1200–5% Bentone 34 sample, which had a maximum operating temperature of 170°.

A 3-min hold period was included in the chromatographic oven temperature-profile program since a 5-cm column is not a good approximation to a thin screen. This hold period more closely normalizes the 5-cm column to the thickness of the screen. Any preconcentrator material that releases the collected explosives vapors during the 3-min hold period would probably not be useful as a screen coating for practical detection applications.

The release temperature of EGDN and 2,4-DNT with each of the preconcentrator materials was measured. This release temperature was read directly from the chromatogram.

RESULTS

The results of the saturation-time and temperature-release tests are summarized in Table III. For the saturation test, only the data on EGDN vapors are reported since the low partial pressure of the 2,4-DNT vapors leads to exceedingly long saturation times. The saturation time of the columns provides a measure of the holding power of each candidate preconcentrator material. A longer saturation time for a particular material therefore indicates that the material has the capacity to retain a large amount of explosives vapor.

TABLE III

SUMMARY OF TEST RESULTS

Material	*Total weight (mg)*	*EGDN saturation time (min)*	*Mass-normalized saturation time (min/mg coating)*	*Release temp. (°C)*	
				EGDN	*DNT*
3% OV-101	47.2	5	3.5	50	99
5% OV-275	51.2	58	22.7	86	151
5% DEGS-PS	54.2	55	20.3	50	134
5% Carbowax 20M-TPA	54.1	103	38.1	50	115
Tenax GC	24.9	120	4.8	136	194
Porapak Q	73.0	450	6.2	155	225
Activated charcoal	71.0	~5750	~80	>225	>225
5% SP-1200–5% Bentone 34	49.8	91	18.3	113	165
Ultrabond 20M	52.1	3	-	50	—

The mass-normalized saturation times are included in Table III to illustrate the difference in application of certain materials. In canister or environmental sampling applications, porous polymers such as Tenax GC have proven to be excellent preconcentrating materials. However, this material would probably not be suitable for screen applications in which consideration must be given to collection efficiency *versus* weight of material since it has a relatively low mass-normalized saturation time (4.8 min/mg coating). In contrast, several tested materials exhibited efficiencies that are four to five times that of Tenax GC and could be used for coating a screen.

Release temperatures for both the EGDN vapors and the 2,4-DNT vapors are also shown in Table III. The release characteristics of each of the candidate materials are as important in the design of a practical vapor preconcentrator as are the collection characteristics. None of the candidate materials exhibited any degradation of the explosives molecules upon release and equal area peaks were observed for all materials except those which "bleed," *i.e.*, give a broad background, at 50°. Bleeding materials which have a release temperature of 50° or lower were judged to be poor candidates for use as preconcentrators. Activated charcoal did not release absorbed explosives vapors even when heated to 300° and consequently is, for all practical purposes, a poor explosives vapor preconcentartor material in heat release applications.

CONCLUSION

Analysis of the data summarized in Table III leads to the conclusion that the

best candidate material of those tested for use as a thin-screen preconcentrator of EGDN and 2,4-DNT vapors from 100 to 1000 l, high-flow air samples is OV-275. OV-275 exhibited good collection properties at its 22.7 min/mg mass-normalized saturation time and desirable release characteristics in a warm (*ca.* 150°) release temperature. In addition, OV-275 takes the form of a highly viscous liquid, which is a practical form for the desired thin-screen geometry.

A further experiment was performed to verify this conclusion. A "column" was constructed for the HP-5840A gas chromatograph that consisted of a stainless-steel screen (6.35 mm O.D., 60 mesh) coated with 0.5 mg of OV-275. This screen was pressed into a thin-walled, stainless-steel tube (6.35 mm O.D.). The chromatographic parameters in this experiment were the same as those used in the release temperature studies. A 1-μl solution of EGDN, dissolved in acetone to a concentration of 98 mg/l, was injected onto the column. The resulting collection and elevated temperature release parameters were consistent with the findings given in Table III.

REFERENCES

1 P. A. Pella, *J. Chem Thermodyn.*, 9 (1977) 301.
2 J. M. Rosen and C. Dickinson, *Vapor Pressures and Heats of Sublimation of Some High Melting Organic Explosives*, NOLTR 69-67, Naval Ordinance Lab., April 1969.
3 A. D. Coates, E. Freedman and L. P. Kuhn, *Characteristics of Certain Military Explosives*, Report Number 1507, U.S. Army Aberdeen Rea. and Dev. Center, Aberdeen Proving Ground, November 1970.
4 J. H. McReynolds, G. A. St. John and M. Anbar, *Determination of Concentration of Explosive Vapor from Parcels and Letters*, Final Report, U.S. Postal Service Contract No. 74-00810, Stanford Research Institute, Stanford, Calif., January 1975.
5 R. B. Cundall, T. F. Palmer and C. C. Wood, *Chem. Soc., Farad. Trans. I*, 74 (1978) 6, 1339.
6 D. C. Leggett, *J. Chromatogr.*, 133 (1977) 83.
7 L. W. Hrubosh, R. L. Morrison, R. W. Ryan, E. G. Walter and M. M. Fulk, *Study of Conventional Preconcentration Techniques for Explosive Vapors*, Lawrence Livermore Laboratory Report No. UCID-16766, Livermore, Calif., August 1973.
8 J. Adams, K. Menzies and P. Levins, *Selection and Evaluation of Solvent Resins for the Collection of Organic Compounds*, Arthur D. Little, Inc., EPA-600/7-77-044, April 1977.
9 *Supelco Catalog No. 12*, Supelco, Inc., Supelco Park, Bellefonte, Pa., 49-51.
10 P. A. Pella and R. M. Mills, *A Trace Vapor Generator for Testing Explosives Vapor Detectors*, Law Enforcement Standards Program Report No. LESP-RPT-0604.00, National Bureau of Standards, Washington, D.C., March 1977.

Journal of Chromatography, 176 (1979) 43-53
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,873

EXPERIMENTAL STUDY OF SELECTIVITY AND COLUMN EFFICIENCY IN CLATHRATE CHROMATOGRAPHY USING WERNER COMPLEXES AS CLATHRATE HOST COMPONENTS

I. RELATIONSHIP BETWEEN SELECTIVITY OF $Ni(NCS)_2$(4-METHYLPYRIDINE)$_4$ · GUEST CLATHRATE SORBENTS AND COMPOSITION OF THE MOBILE PHASE

JANUSZ LIPKOWSKI, MARIA PAWŁOWSKA and DANUTA SYBILSKA
Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw (Poland)
(First received October 10th, 1978; revised manuscript received March 9th, 1979)

SUMMARY

The chromatographic properties of the clathrates formed by $Ni(NCS)_2$ (4-methylpyridine)$_4$, treated as representative of a large group of Werner MeX_2A_4 complexes able to form clathrates, were studied. As the compounds to be separated, *o*-, *m*- and *p*-isomers of dinitrobenzene and bromonitrobenzene were used. It was found that the volume of the channels in the zeolite-like Ni $(NCS)_2$(4-methylpyridine)$_4$ structure can vary by about 50%, owing to dilatation of the crystal structure related to the composition of the guest, and can be easily controlled by using a suitable composition of the mobile phase. The selectivity of chromatographic sorption as a function of dilatation–contraction of the clathrate structure and competitive clathration of the mobile phase components (elution power) is discussed.

INTRODUCTION

Since the first paper on the separation of isomers using clathrate-type compounds as stationary phases was published[1], many different mixtures have been separated by this chromatographic method[1-5]. Trial and error approaches have usually been made in order to find suitable conditions for selectivity and the column parameters necessary for the satisfactory solution of a given separation problem. Recently, knowledge of the physico-chemical properties and the structure of several $Ni(NCS)_2$(4-MePy)$_4$* clathrates was greatly increased as is described below in more detail. This progress made possible a detailed study of correlations between the structural properties of the clathrates and their chromatographic parameters.

The aim of this work was to study the correlations mentioned above from the

* 4-MePy = 4-methylpyridine.

point of view of optimization and design of liquid chromatographic separations of isomers using clathrate-forming Werner complexes.

EXPERIMENTAL

Reagents

All reagents and solvents were of analytical-reagent grade. 4-Methylpyridine contained less than 0.5% of 3-methylpyridine as the main impurity.

Apparatus

The detection system used was the Kemula apparatus[6] for chromatopolarography combined with a Radelkis OH 101 d.c. polarograph. X-ray diffractograms were taken using a Rigaku-Denki powder diffractometer (Cu K_{α}).

Preparation of the sorbents

The clathrate sorbents, which are virtually insoluble in the mobile phases used, were precipitated by simultaneous, slow pouring of aqueous $Ni(NCS)_2$ (0.2 *M*) and methylpyridine into a suitable mixture of organic solvent, NH_4SCN and water. The precipitate was then kept in the mother liquor at 25 ± 0.1° for 24 h before use. Acetone (Ac), ethanol(Et), *n*-propanol (Prop) and ethylene glycol (Glyc) were used as organic solvents. Their concentrations (percent by volume) and the 4-methylpyridine content in the mobile phase (mother liquor) are indicated in the text (see Tables I and II) in the form, *e.g.*. Ac 27/3.4, denoting $Ni(NCS)_2(4\text{-}MePy)_4 \cdot G^*$ sorbent equilibrated with aqueous acetone (27%, v/v) containing (3,4% ,v/v) of 4-methylpyridine.

Chromatographic experiments

Glass columns (40 × 6 mm I.D.) were prepared by slurry packing; the particle size was ≤15 μm. Mother liquors were always used as mobile phases and the flow-rate of the mobile phase was 10 ml/h.

The capacity factors (k') were calculated from the equation

$$k' = \frac{V_R - V_0}{V_0}$$

where V_R is the retention volume and V_0 is the volume of the mobile phase in the column calculated as the retention volume of furazol (N-5-nitro-2-furfurylidene-3-amine-2-oxazolidone).

Analytical determinations

The procedure for phase analysis of the substances used has been published previously[7]. Lattice parameters of the clathrates were determined from X-ray diffractograms and refined by using the least-squares method.

The density of the clathrates was measured by pyknometry, slightly modified to avoid weighing of dried material; it has been demonstrated[8] that drying affects the composition and structure of the clathrates.

* G = Guest.

RESULTS AND DISCUSSION

Clathrate sorbents

As shown by X-ray powder diffraction patterns tetragonal $I4_1/a$, the β-phase of $Ni(NCS)_2(4\text{-}MePy)_4$, results if the procedure described under *Preparation of the sorbents* is followed, with the use of 4-methylpyridine at concentrations of 1.5–7.5% and the organic solvent at concentrations of 18–60%. The β-structure, recently determined by De Gil and Kerr[9], is of the "zeolite" or channel type. However, this structure, which consists of discrete host complex molecules packed together by means of weak Van der Waals forces, is "sensitive" to sorption of the guest, *i.e.*, it swells on absorbing more guest[8]. It can be seen from the data in Table I that the lattice parameters of the sorbent can easily be changed over a wide range simply by varying the concentrations of 4-methylpyridine and organic solvent in the liquid phase, which is in an equilibrium state with respect to the solid clathrate. The differences, which reach about 17% of the molar volume of the clathrate, are very significant if expressed as differences in the molar "free volume" of the sorbent, *i.e.*, the volume not occupied by the host. If the molar volume of the host in its non-clathrate α-modification (430 cm^3) is subtracted from the molar volume of the clathrate sorbents, the free volume varies from 47 to 90 cm^3/mole. It seems that there is no significant change in the c/a axial ratio and the swelling of the clathrate can be regarded as "proportional" in all three dimensions.

From studies of sorption isotherms in the β-$Co(NCS)_2(4\text{-}MePy)_4$ phase, Allison and Barrer[10] found an analogy to the water–montmorillonite system. Stepwise or inflected isotherms were obtained and a modified Langmuir model proposed:

$$\theta = \frac{p}{K + p}\left[\frac{N_1 + F(x)}{N_1 + N_2}\right]$$

where

K = equilibrium constant;
p = external pressure of the sorbate;
N_1 = number of sites initially present;
N_2 = the maximum extra number of sites generated;
$F(x)$ = an equilibrium number of sites generated, dependent on external pressure of the sorbate and temperature.

It seems reasonable to assume a similar model for sorption equilibria in the system β-$Ni(NCS)_2(4\text{-}MePy)_4$ + 4-methylpyridine + organic solvent. At high solvent but low 4-methylpyridine concentrations in the mobile phase there is one molecule of the solvent absorbed per molecule of the host. At higher concentrations of 4-methylpyridine there is one molecule of 4-methylpyridine absorbed in addition. Within these limiting values, the equilibria are more complex. For example, in the ranges of 2.3–7.6% of 4-methylpyridine and 27–60% of acetone, one molecule of 4-methylpyridine substitutes *ca.* 0.8 molecule of acetone[11]. This can be interpreted on the basis of the model described above, *e.g.*, five molecules enter into sites occupied by five molecules of acetone substituting them, but at the same time an additional site is generated and one molecule of acetone remains absorbed. Experimentally found data for the mean molecular weight of the guest in the clathrate (Table II) serve to define the overall sorption of all components of the mobile phase.

TABLE I

LATTICE PARAMETERS AND POROSITY OF β-Ni $(NCS)_2$ $(4\text{-MePy})_4 \cdot G$ CLATHRATES
G = 4-MePy + (acetone or ethanol or *n*-propanol or ethylene glycol).

Clathrate sorbent	*a (Å)* (±0.02)	*c (Å)* (±0.02)	*Molar volume (cm³)* (± 1.6)	*Molar volume of pores (cm³)* (± 1.6)
Ac 18/1.5	17.01	23.22	505.8	75.8
Ac 18/2.3	17.04	23.31	509.3	79.3
Ac 18/3.4	17.10	23.36	514.4	84.4
Ac 18/5.1	17.10	23.40	515.2	85.2
Ac 18/7.6	17.17	23.45	520.6	90.6
Ac 27/1.5	16.82	22.59	481.0	51.0
Ac 27/2.3	16.87	22.78	487.9	57.9
Ac 27/3.4	16.99	23.23	504.6	74.6
Ac 27/5.1	17.12	23.41	516.7	86.7
Ac 27/7.6	17.13	23.41	517.4	87.4
Ac 40/1.5	16.77	22.65	479.7	49.7
Ac 40/2.3	16.88	22.88	491.0	61.0
Ac 40/3.4	16.87	23.11	495.4	65.4
Ac 40/5.1	17.02	23.18	505.7	75.7
Ac 40/7.6	17.17	23.48	521.0	91.0
Ac 60/1.5	16.75	22.69	478.2	48.2
Ac 60/2.3	16.78	22.72	481.8	51.8
Ac 60/3.4	16.79	22.66	480.5	50.5
Ac 60/5.1	16.88	22.85	490.4	60.4
Ac 60/7.6	17.07	23.27	510.4	80.4
Et 18/1.5	17.09	23.26	511.6	81.6
Et 18/2.3	17.11	23.29	513.4	83.4
Et 18/3.4	17.18	23.41	520.1	90.1
Et 18/5.1	17.19	23.45	521.8	91.8
Et 18/7.6	17.15	23.45	519.6	89.6
Et 27/1.5	17.07	23.14	507.5	77.5
Et 27/2.3	17.11	23.22	512.0	81.0
Et 27/3.4	17.15	23.28	515.5	85.5
Et 27/5.1	17.12	23.28	513.9	83.9
Et 27/7.6	17.14	23.26	514.7	84.7
Et 40/1.5	16.99	23.02	500.4	70.4
Et 40/2.3	17.02	23.08	503.3	73.3
Et 40/3.4	17.08	23.17	509.1	79.1
Et 40/5.1	17.10	23.17	510.2	80.2
Et 40/7.6	17.12	23.22	512.3	82.3
Et 60/1.5	17.00	23.06	501.6	71.6
Et 60/2.3	17.09	23.12	508.4	78.4
Et 60/3.4	17.13	23.13	511.1	81.1
Et 60/5.1	17.09	23.11	508.4	78.4
Et 60/7.6	17.08	23.17	509.1	79.1
Glyc 18/3.4	17.14	23.40	517.4	87.4
Glyc 18/7.6	17.17	23.46	520.7	90.7
Glyc 27/3.4	17.03	23.29	508.5	78.5
Glyc 27/7.6	17.16	23.47	520.8	90.8
Glyc 40/3.4	17.04	23.35	510.1	80.1
Glyc 40/7.6	17.21	23.34	520.2	90.2

TABLE I (*continued*)

Clathrate sorbent	*a* (Å) (±0.02)	*c* (Å) (±0.02)	*Molar volume* (cm^3) (±1.6)	*Molar volume of pores* (cm^3) (±1.6)
Prop 18/3.4	17.09	23.18	509.8	79.8
Prop 18/7.6	17.14	23.39	517.5	87.5
Prop 27/3.4	16.93	22.86	493.4	63.4
Prop 27/7.6	17.11	23.23	511.8	81.8
Prop 40/3.4	16.82	22.78	485.2	55.2

Clathrate selectivity as a function of eluent composition

On the basis of the X-ray diffraction data described above, one might expect a great dependence of the chromatographic properties of the clathrates on their lattice constants, that is, the size of the cages. This is indeed so, as illustrated in Fig. 1.

It can be seen (Figs. 2 and 3) that the mobile phase composition influences not only the absolute, but also the relative capacity factors and that the selectivity ($\alpha = k'_2/k'_1$) is related to the eluent composition.

A special feature of these diagrams is that the capacity factor (k') plotted against 4-methylpyridine concentration has a maximum at a value of the latter which depends on the solvent concentration. In other words, there is an optimum for chromatographic separation that may be related to structural factors. However, there is no obvious relationship between the size of the cage and the capacity factor that might

TABLE II

DENSITY (d), MOLECULAR WEIGHT (M_{Cl}) OF CLATHRATE AND MEAN MOLECULAR WEIGHT (M_G) OF THE GUEST IN CLATHRATE

Clathrate sorbent	d (g/cm^3)	M_{Cl}	M_G
Ac 18/1.5	1.2962	655.6	108.4
Ac 18/7.6	1.2687	660.5	113.3
Ac 27/3.4	1.2164	613.8	66.6
Ac 27/5.1	1.2561	649.0	71.8
Ac 27/7.6	1.2578	650.8	103.6
Ac 40/3.4	1.2107	559.8	52.6
Ac 40/5.1	1.2365	625.3	78.1
Ac 60/1.5	1.2396	592.8	45.6
Ac 60/3.4	1.2618	606.3	59.1
Ac 60/7.6	1.2104	617.8	70.6
Et 18/1.5	1.2585	643.8	96.6
Et 18/7.6	1.2921	671.4	124.2
Et 27/1.5	1.2337	626.1	78.9
Et 27/2.3	1.2643	647.3	101.1
Et 27/3.4	1.2474	643.0	95.8
Et 27/5.1	1.2368	635.6	88.4
Et 40/2.3	1.2477	627.9	80.7
Et 40/3.4	1.2747	648.9	101.7
Et 60/1.5	1.1949	599.4	52.2
Et 60/7.6	1.1949	608.1	60.9

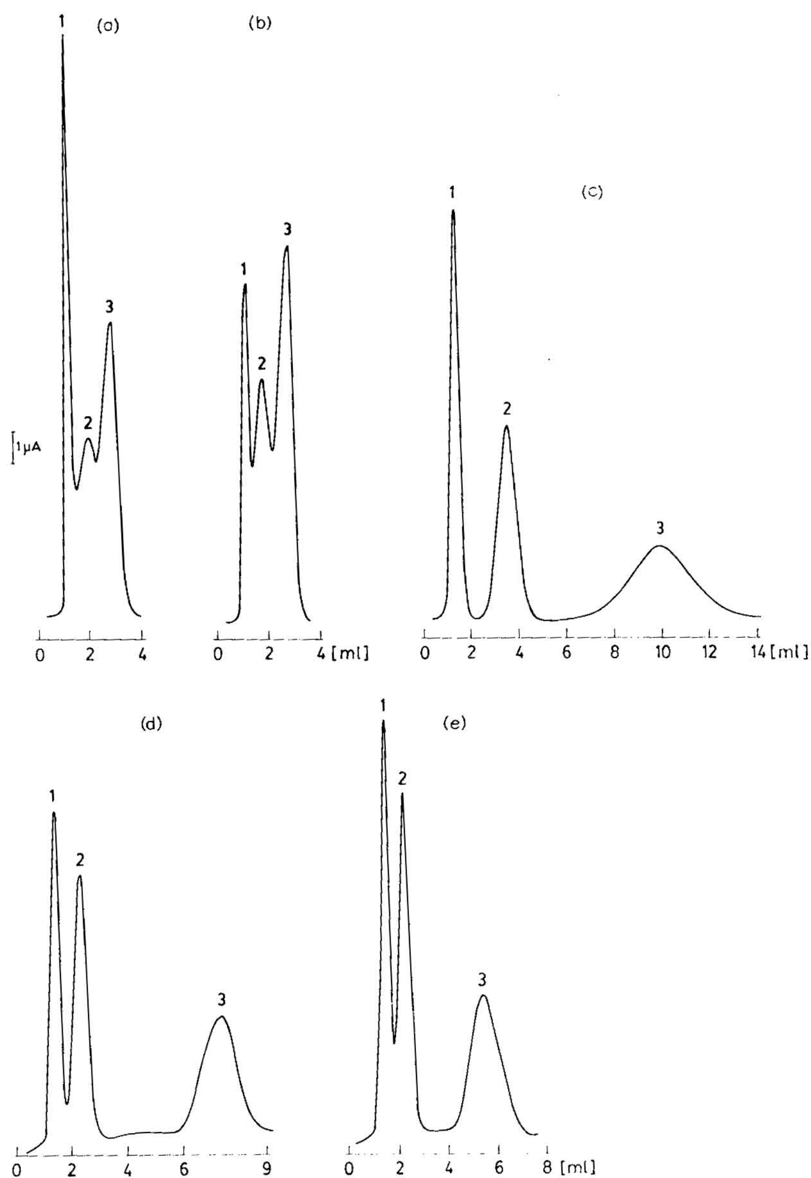


Fig. 1. Dependence of chromatographic separation of dinitrobenzene isomers on clathrate structure and mobile phase composition. Peaks 1, 2 and 3 represent *o*-, *m*- and *p*-dinitrobenzene, respectively. Column, 40 × 6 mm I.D.; $d_p \leq 15\,\mu m$; flow-rate, 10 ml/h. (a) Ac 27/1.5, $V_{mol} = 481$ cm^3; (b) Ac 27/2.3, $V_{mol} = 488$ cm^3; (c) Ac 27/3.4, $V_{mol} = 505$ cm^3; (d) Ac 27/5.1, $V_{mol} = 516$ cm^3; (e) Ac 27/7.6, $V_{mol} = 517$ cm^3.

serve for selecting an optimal sorbent. The other factor that has to be involved is the competition of the mobile phase components with respect to the clathration of isomers to be analysed. The following discussion is based on the assumptions that:

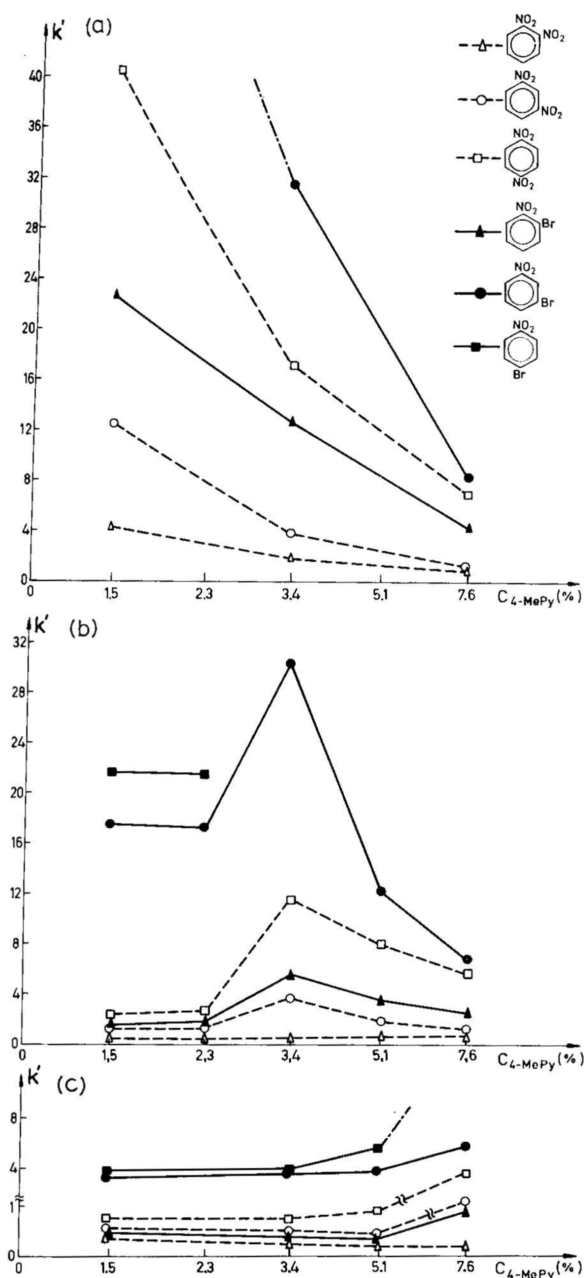


Fig. 2. Dependence of k' on mobile phase composition. Column, 40 × 6 mm I.D.; $d_p \leqslant 15\ \mu m$; flow-rate, 10 ml/h. (a) 18% acetone; (b) 27% acetone; (c) 40% acetone.

(a) dilatation of the clathrate host structure increases the capacity factor for the absorption of benzene derivatives and

(b) absorption of 4-methylpyridine and an aliphatic solvent (the components

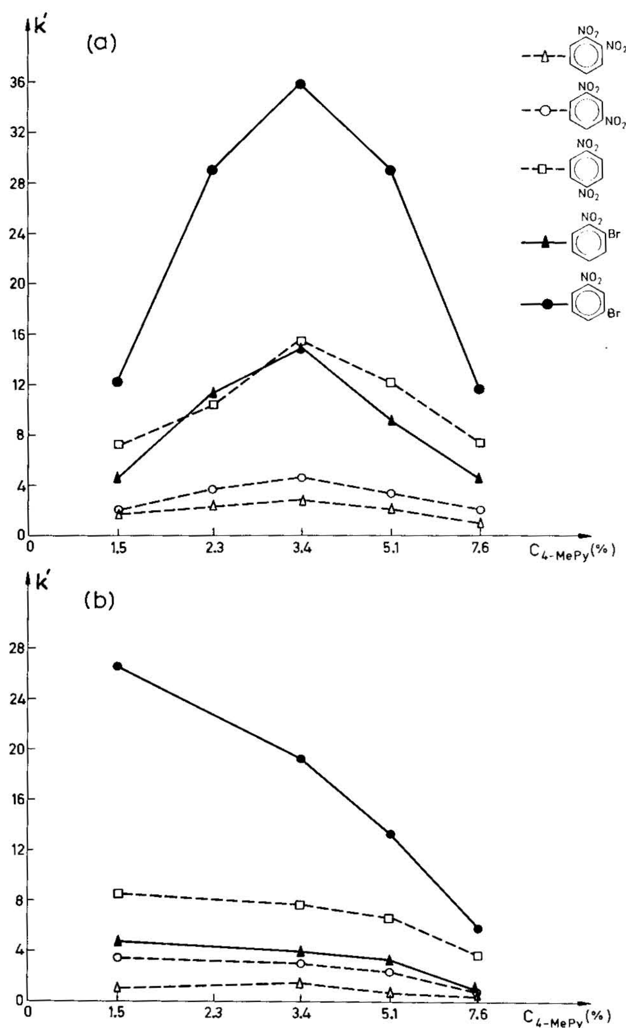


Fig. 3. Dependence of k' on mobile phase composition. Column, 40 × 6 mm I.D.; d_p ≈ 15 μm; flow-rate, 10 ml/h. (a) 18% ethanol; (b) 40% ethanol.

of the mobile phase) compete with the absorption of the compounds being analysed and causes a decrease in k'.

The example in Fig. 2 demonstrates these two effects. Maxima are present at $c_{solv} = 27\%$ and $c_{4\text{-MePy}} = 3.4\%$ (Fig. 2b). At $c_{solv} = 18\%$ the maxima are presumably shifted to $c_{4\text{-MePy}} < 1.5\%$ (Fig. 2a) and at $c_{solv} = 40\%$ to $c_{4\text{-MePy}} > 7.6\%$ (Fig. 2c). At any given concentration of the solvent, the cage size increases with increasing concentration of 4-methylpyridine. At the same time, competitive clathration of 4-methylpyridine becomes more important, because of its higher concentration in the liquid phase. Thus, in the increasing part of the $k' = k'$ ($c_{4\text{-MePy}}$) relationship, the dominating factor is lattice dilatation favourable for absorption of the analysed guests. It might be suggested that dilatation is equivalent to the generation of absorption sites.

After the maximal number of the sites has been reached, competitive clathration of 4-methylpyridine becomes predominant and k' decreases as $c_{4\text{-MePy}}$ increases. On going to higher concentrations of aliphatic solvents, it should be noted that lattice contraction occurs (see Table I). To generate absorption sites a higher 4-methylpyridine concentration is necessary, *e.g.*, with 40% acetone a higher concentration of 4-methylpyridine is required than at 27% acetone. Similarly, at 18% acetone the maximal number of absorption sites appears at $c_{4\text{-MePy}} < 1.5\%$.

Let us assume that sorption of the guest (G) follows a substitution mechanism:

$$\mathrm{KP} + \mathrm{G} \rightleftharpoons \mathrm{KG} + \mathrm{P} \text{ or } \mathrm{KR}_x + \mathrm{G} \rightleftharpoons \mathrm{KG} + x\mathrm{R}$$

where KP, KR_x and KG denote clathrates with 4-methylpyridine (P), solvent (R) or sorbate (G) as the guest, respectively. Then, equilibrium constants can be written as

$$c_{PG} = \frac{[\mathrm{KG}]\,[\mathrm{P}]}{[\mathrm{KP}]\,[\mathrm{G}]} \quad \text{or} \quad c_{RG} = \frac{[\mathrm{KG}]\,[\mathrm{R}]^x}{[\mathrm{KR}_x]\,[\mathrm{G}]}$$

Taking into account that

$$\frac{[\mathrm{KG}]}{[\mathrm{G}]} = k' \quad \text{and} \quad [\mathrm{KP}] + [\mathrm{KR}_x] = [\mathrm{K}]$$

the following equations can be derived:

$$k' = \frac{c_{RG} \cdot \frac{1}{\kappa} \cdot [\mathrm{K}]}{[\mathrm{P}] + \frac{1}{\kappa} \cdot [\mathrm{R}]^x} \quad \text{or} \quad k' = \frac{c_{PG}\,[\mathrm{K}]}{[\mathrm{P}] + \frac{1}{\kappa} \cdot [\mathrm{R}]^x}$$

where $\kappa = [\mathrm{KP}]\,[\mathrm{R}]^x/[\mathrm{KR}_x]\,[\mathrm{P}]$ is the equilibrium constant for substitution of solvent (R) with 4-methylpyridine (P) in the clathrate.

Comparing the two expressions for k', it should be noted that the relationship $k' = k'([\mathrm{P}], [\mathrm{R}])$ is essentially of the same type in both instances; c_{RG}, c_{PG}, κ and K are constant. The equations can be considered as analogous to Snyder's[12] and Soczewiński's[13] equations:

$$k' = \frac{B}{[x]^n}$$

but the elution factor in clathrate chromatography is the sum of $[\mathrm{P}] + 1/\kappa \cdot [\mathrm{R}]^x$.

If only κ and x are really constant, the experimental results for $k' = k'([\mathrm{P}], [\mathrm{R}])$ are relatively clear and understandable, as in Fig. 2. Moreover, the curves in Figs. 2a and 3b, even if they refer to different solvents, are in fact similar, because they correspond to similar values of $1/\kappa \cdot [\mathrm{R}]^x$ in both instances[11].

However, the problem is more complex. x may even assume negative values, *i.e.*, absorption of 4-methylpyridine causes additional sorption and not elimination

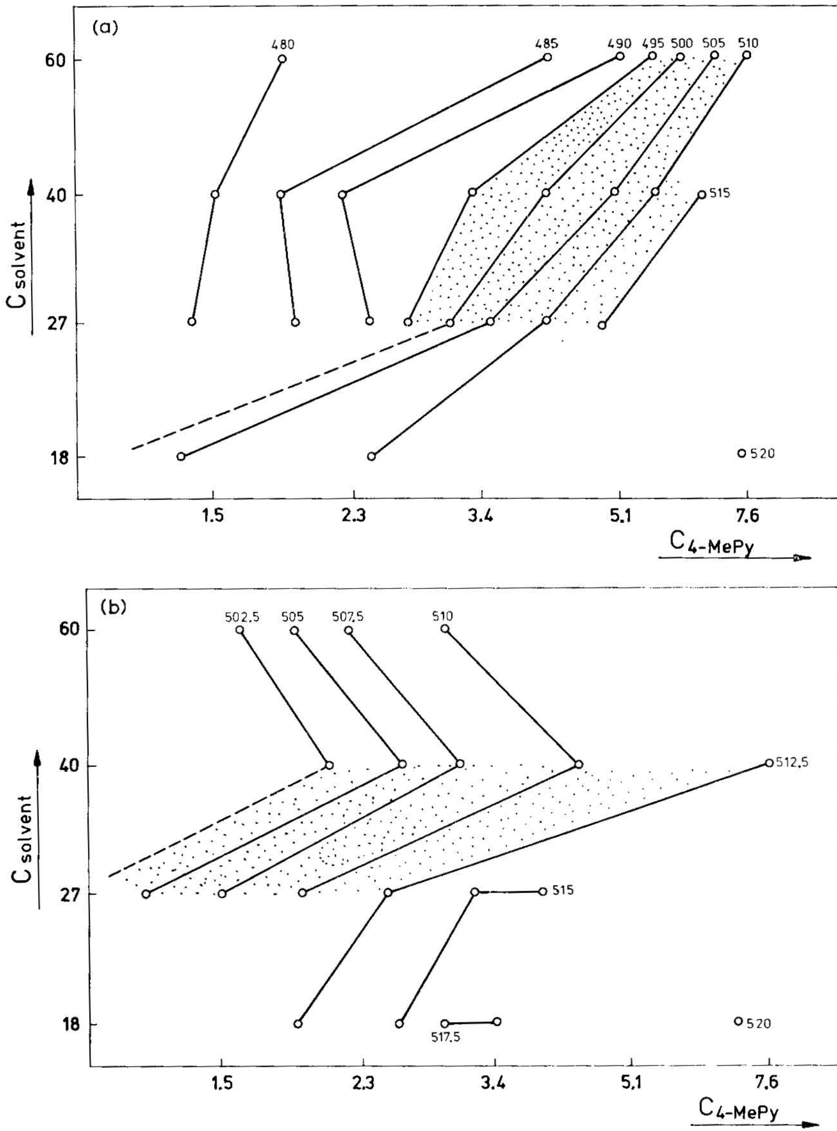


Fig. 4. Layer diagram of molar volume of clathrate sorbents plotted against 4-methylpyridine ($c_{4\text{-MePy}}$) and solvent (c_{solv}) concentrations in the mobile phase. In the shaded regions a substitution mechanism, $KR_x + P \rightarrow KP + xR$, is valid; x is approximately constant (see text). (a) solvent = acetone, $x \approx 0.8$; (b) solvent = ethanol, $x \approx 2$.

of the solvent in the clathrate, because of lattice dilatation. The equations given above for k' have no meaning in such instances. The applicability of the above considerations is illustrated in Fig. 4.

If the equilibrium were of the type $KR_x + P \rightleftharpoons KP + xR$, the molar volumes should be constant and appear as parallel straight lines on the diagrams. This is the case only within narrow limits and the explanation given above is valid only in these

ranges. Finally, it must be mentioned that the solubility of the sorbate in the mobile phase influences the k' values: a higher concentration of organic solvent in the mobile phase results in smaller k' values. This effect is not selective, of course, and appears as a uniform lowering of the $k' = k'([P])$ curves on going to higher [R] values.

Summarizing the above considerations, the following conclusions can be drawn:

(1) Selectivity of clathration is determined by the host structure in the clathrate, defining shape of the cage–absorption sites. The β-structure studied in this work is "para-selective" with respect to mixtures of isomers of disubstituted benzene derivatives.

(2) The number of effective absorption sites in the clathrate is greatly dependent upon the lattice parameters of the β-structure, which may be varied by clathration of the mobile phase components. By controlling the composition of the mobile phase by varying the concentration of 4-methylpyridine and/or organic solvent, one can produce sorption of the mobile phase components in order to obtain a clathrate sorbent with the desired lattice constants and hence sorption capacity.

(3) Adjustment of the absorption sites in clathrates by controlling the mobile phase composition must involve simultaneous variation of the "elution power" of the mobile phase. Two factors contribute to the elution power: competitive clathration of the mobile phase components and the solubility of the sorbate in the liquid phase. The former can be written as a sum, $[P] + 1/\kappa \cdot [R]^x$; the latter has not been studied in this work as a non-selective, typical effect in liquid chromatography.

In conclusion, β–$Ni(NCS)_2(4\text{-}MePy)_4$ clathrate sorbents have valuable features of high selectivity and versatility of sorption. Optimization of the operating parameters in these liquid chromatographic systems will be the subject of a forthcoming paper.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Prof. W. Kemula for stimulating discussions of this problem. This work was supported within project 03.10.

REFERENCES

1 W. Kemula and D. Sybilska, *Nature (London)*, 185 (1960) 237.
2 D. Sybilska, *Proc. VI Int. Symp. on Chromatography and Electrophoresis*, Presses Academiques Europeennes, Brussels, 1971, pp. 212–221.
3 W. Kemula and D. Sybilska, *Anal. Chim. Acta*, 38 (1967) 97.
4 W. Kemula, D. Sybilska and K. Duszczyk, *J. Microchem.*, 11 (1966) 296.
5 W. Kemula, Z. Borkowska and D. Sybilska, *Monatsh. Chem.*, 103 (1972) 860.
6 W. Kemula, in L. Zuman and I. M. Kolthoff (Editors), *Progress in Polarography*, Wiley-Interscience, New York, 1962, p. 397.
7 W. Kemula, J. Lipkowski and D. Sybilska, *Rocz. Chem.*, 48 (1974) 3.
8 J. Lipkowski and S. Majchrzak, *Rocz. Chem.*, 49 (1975) 1655.
9 E. R. de Gil and T. S. Kerr, *J. Appl. Crystallogr.*, 10 (1977) 315.
10 S. A. Allison and R. M. Barrer, *J. Chem. Soc.*, (1969) 1717.
11 M. Pawłowska, *Thesis*, Warsaw, 1978.
12 L. R. Snyder, *Anal. Chem.*, 46 (1974) 1384.
13 E. Soczewiński, *J. Chromatogr.*, 130 (1977) 23.

Journal of Chromatography, 176 (1979) 55–64
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,863

N-CHLOROMETHYLPHTHALIMIDES AS DERIVATIZATION REAGENTS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

W. LINDNER

Department of Pharmaceutical Chemistry, University of Graz, Graz (Austria)

and

W. SANTI*

Analytical Research and Development, Pharmaceutical Division, Sandoz Ltd., 4002 Basle (Switzerland)

(Received February 26th, 1979)

SUMMARY

A new class of substances is described, the N-chloromethylphthalimides, which can be used as UV-sensitive reagents for the formation of derivatives suitable for high-performance liquid chromatography (HPLC).

The highly reactive chlorine in the N-methyl group reacts quantitatively with alkali metal or ammonium salts of CH-, OH- and NH-acid compounds. The salts of the free acids can be formed by direct addition of triethylamine to the reaction mixture. Hence the reagent is suitable for fast pre-column derivatization of free acids. Aprotic solvents are suitable reaction media. Acetonitrile is used with preference, as the reaction solution does not absorb at 254 nm and can be analysed directly by using HPLC.

If a crown ether is used as a phase transfer catalyst, alkali metal salts of carboxylic acids can be quantitatively converted within 15 min at 60°. The applications of this group of reagents in derivatization are illustrated by examples of mono- and dicarboxylic acids and barbiturates.

INTRODUCTION

The need to produce derivatives for high-performance liquid chromatography (HPLC) which are readily detectable by photometric methods has prompted numerous studies in recent years. Lawrence and Frei[1] and Blau and King[2] reviewed the UV-sensitive and fluorescence-sensitive reagents which were then in use, together with their fields of application. It is evident that the assay of compounds in trace amounts is often possible only if specific, selective derivatization reactions are used, as the detectability can be appreciably improved and the derivatization step usually serves also as a clean-up procedure in the analysis of a complex matrix.

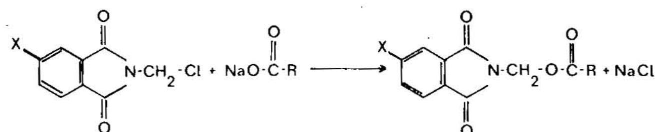
This paper examines the suitability of N-chloromethylphthalimides for use as derivatization agents in HPLC, with special reference to their selectivity and reactivity.

* To whom correspondence should be addressed.

In Table I spectrophotometric data and references are listed for the substances studied, namely N-chloromethylphthalimide (ClMPI) and N-chloromethyl-4-nitrophthalimide (ClMNPI). The isomer of N-chloromethylphthalimide, N-chloromethylisatin (ClMIS), was also studied, as its chemical properties are very similar to those of the imide. The halogen atom of the N-methyl group in all of these compounds is very mobile and as a result it reacts with various proton-active groups.

TABLE I

UV-SPECTROSCOPIC DATA FOR N-CHLOROMETHYLPHTHALIMIDES



ClMPI : X — H
ClMNPI: X — NO_2

Compound	*Abbreviation*	λ_{max} *(nm) in acetonitrile*	$\varepsilon_{\lambda max}$	$\varepsilon_{254\ nm}$	*References*	*M.p. (°C)*
N-Chloromethyl-phthalimide	ClMPI	224	18,700	1390	3–6	135
N-Chloromethyl--4-nitrophthalimide	ClMNPI	235	23,900	9500	4	142
1-Chloromethylisatin	ClMIS	240			6–10	100

Table II gives a brief survey of the reactions in which this type of compound can take part. Details of reaction velocities and yields are not given, as the data in the literature are not readily comparable.

TABLE II

FUNCTIONAL GROUPS REACTING WITH N-CHLOROMETHYLPHTHALIMIDES AND N-CHLOROMETHYLISATIN

Functional group	*Type of reagent*	*References*
Carboxyl	ClMIS	7–9
	ClMPI	3, 5, 6
CH-acid	ClMIS	7
	ClMPI	3
NH-acid	ClMIS	7–9
Amine	ClMIS	7, 11
Hydroxyl	ClMIS	7, 12

As for the reaction mechanism, it can be stated that the N-chloromethylphthalimides, as well as N-chloromethylisatin in general react only with the salts of CH-, NH- and OH-acid compounds. For preference the sodium salts are used[7–9], although ammonium salts react satisfactorily in certain instances[5]. Any aprotic solvent will serve as the reaction medium. The numerous reactions open to this class of compounds and their wide field of application in analysis can be seen in Table II.

The usefulness of these derivatization reagents for carboxyl groups will now be

examined in detail, and the reaction kinetics, with and without catalysts, will also be discussed. In a further section, the formation of barbiturate and phenol derivatives will be described.

Determination of carboxylic acids as phthalimidomethyl or 4-nitrophthalimidomethyl esters using HPLC

With optical detectors, the HPLC assay of carboxylic acids with poor chromophores is of very low sensitivity. By derivatization of the carboxyl groups to esters with UV-sensitive reagents, the detectability of the acids can be greatly improved and, moreover, the high polarity of the acids is decreased, so that, in addition, the derivatives generally possess better chromatographic properties.

Acid derivatives which are sensitive to UV include the corresponding benzyl[13], *p*-nitrobenzyl[14,15], phenacyl[15,16], *p*-nitrophenylacyl[17,18], *p*-bromophenacyl[18–20] and 2-naphthacyl[21] esters. All of these derivatives have been chromatographically investigated and good HPLC separations of the acid–ester mixtures were achieved in every instance. By this method it also proved possible to separate *cis–trans* isomers such as C_{18}' and C_{18}'' as well as isomers with the double bond in a different position[19].

It can be seen from Table I that, as expected, the nitration of ClMPI caused a shift of λ_{max} towards the visible region of the spectrum. The molar extinction coefficient, ε, is higher for ClMNPI than for ClMPI at λ_{max}, but at 254 nm, the wavelength which is most commonly used for fixed-wavelength detectors in HPLC, ε, for the nitro compound is higher by a factor of 7 and therefore the detectability of the analogous 4-nitrophthalimidomethyl derivatives is correspondingly greater. In this study both reagents were used, although it was realised that in the future preference would be given to ClMNPI because of its greater sensitivity enhancement.

EXPERIMENTAL

HPLC system

All analyses were carried out on a Hewlett-Packard 1010B apparatus with a Hewlett-Packard 1036A fixed-wavelength detector. The columns were HIBAR columns filled with LiChrosorb RP8 of average particle size 7 μm (Merck, Darmstadt, G.F.R.).

Reagents

The solvents were of analytical-reagent grade and were supplied by Merck. They were used without further purification. The crown ether 18-Crown-6-(1,4,7,10,13,16-hexaoxacyclooctadecane) of purum quality was supplied by Fluka (Buchs, Switzerland).

N-Chloromethylphthalimide was synthesized using the method of Böhme *et al.*[3]. The reagent was recrystallised from dry benzene–cyclohexane and could be kept in dry storage at room temperature for several months without decomposition. N-Chloromethyl-4-nitrophthalimide was synthesized by a procedure analogous to that described by Böhme and co-workers[3,4], and 4-nitrophthalimide was produced by the method of Huntress and Shriner[22]. The yields obtained corresponded with the data in the literature.

Some of the fatty acids and dicarboxylic acids were of analytical-reagent grade

and others of purum quality. All of the barbiturates were of the degree of purity required for pharmaceutical purposes.

Derivatization reaction

Method A (used for acid concentrations of 0.5–10.0 mM). The sample of a non-volatile, organic acid is dissolved in methanol or diethyl ether and carefully evaporated to dryness in a 50-ml reaction vessel. The residue is taken up in an approximately 3 molar excess of triethylamine (TEA) and 20 ml of acetonitrile (or dimethylformamide), and a 3 molar excess of ClMPI or ClMNPI is then added.

The vessel is closed and kept at 60° (in a drying oven) for 1 h and the contents are then added with stirring to about 100 ml of water at room temperature. A copious precipitate settles out, which is a mixture of excess of reagent and the ester in question. This residue can be freed from the reagent by several crystallizations from benzene–cyclohexane and can be obtained in a form suitable for analysis in this way.

Method B (used for acid concentrations of 0.001–0.5 mM). The samples of non-volatile organic acid, which are dissolved in water, methanol or diethyl ether, are carefully evaporated to dryness in reaction vessels that can be firmly closed (ampoule flasks) of 1 or 5 ml volume. The residue, together with an excess of the alkylating reagent (ClMPI or ClMNPI) and triethylamine (as in method A), is taken up in 0.5–3 ml of acetonitrile. The vessel is firmly closed and kept at 60° for 1 h.

After cooling, the solution can be assayed directly by HPLC; any colourless needles that may precipitate are triethylammonium chloride and do not affect the determination.

Method C (used for acid concentrations of 0.001–0.5 mM). Unless it is already in the form of an aqueous solution of carboxylic acid salts, the sample of organic acids (including volatile acids), dissolved in water or methanol, is neutralized to phenolphthalein with methanolic potassium hydroxide solution. The salt solutions are immediately placed in the reaction vessels and carefully evaporated to dryness.

An excess of alkylating medium and crown ether in a molar ratio of 10:1 in acetonitrile is added to the residue and the reaction vessel is then placed in a drying oven for 1 h at 60° with shaking at intervals. The solution can be assayed directly.

For the derivatization of barbiturates, methods A, B and C are used. Quantitative derivatization of the phenolic hydroxy group may be effected by method C via the alkali metal phenolates which are readily formed using methanolic potassium or sodium hydroxide solution. Method C is to be used for the alkali metal salts.

RESULTS AND DISCUSSION

Derivatization studies

In order to evaluate a derivatization reaction for use in analysis, it is necessary to investigate the rate of reaction and the yield. The reagents tested (ClMPI and ClMNPI) react almost quantitatively ($>97\%$) with both the lower and higher fatty acids and with the dicarboxylic acids (C_2–C_6).

The NH-acid compounds, as represented by the barbiturates, are also quantitatively derivatized. Moreover, the phenolic hydroxyl group can be converted quantitatively into the corresponding ether.

ClMPI and ClMNPI react satisfactorily only with salts of CH-, OH- and NH-

acid compounds; these salts may be the alkali metal or, as in the case of TEA, the ammonium salts. The reaction with alcoholic OH groups does not go to completion under the given conditions (methods A–C) owing to inadequate alcoholate formation, and similarly, conversion is incomplete and variable with primary and secondary amines. Tertiary amines tend to form quaternary ammonium compounds with the chloromethylimides, and in methods A and B this leads to side-reactions with the excess of TEA. The reaction is appreciably slower than that with the acid; nevertheless, if the excess of TEA is too small, it is possible that the derivatization will not reach 100% yield as no further TEA remains for the formation of the required salt. ClMPI and ClMNPI react very slowly with water, which is an advantage as it is not critical if small amounts of water are still present in the samples to be derivatized.

The rate of the esterification or etherification reaction does not depend very much on the reaction medium, provided that it is an aprotic water-miscible solvent. We prefer to use acetonitrile, as this serves also as a component of the mobile phase in HPLC and, moreover, shows no UV-absorption at 254 nm. TEA salts of the acids generally react quantitatively within 30–40 min in acetonitrile at 60°.

As can be seen from Fig. 1, the alkali metal salts react very slowly when the reaction, is not catalysed, so that complete conversion is unlikely to occur. The use of TEA or 4-dimethylaminopyridine (DMAP) greatly accelerates the reaction, probably by promoting solubility.

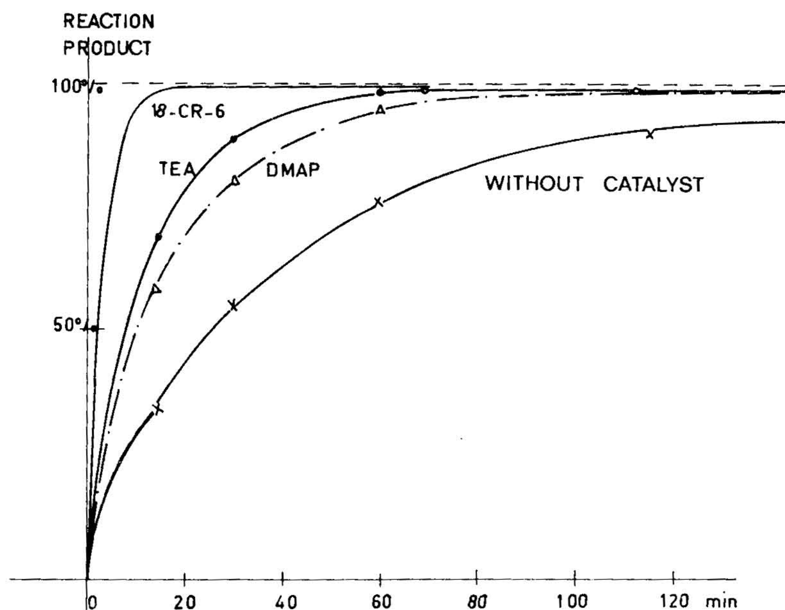


Fig. 1. Reaction rate of sodium acetate with N-chloromethylphthalimide in acetonitrile at 60°.

Phase-transfer catalysis with a crown ether causes the greatest increase in reactivity in these alkylation reactions, and according to Durst and co-workers[20,23] it has likewise proved very effective in the formation of phenacyl esters. After a reaction time of 15 min, the conversion of sodium acetate is virtually complete.

C_2–C_{18} fatty acids, including unsaturated fatty acids, and also the C_2–C_6 dicarboxylic acids, show slight decreases in reactivity as the chain length increases; the long-chain acids react like TEA salts (method B) within 60 min.

At present no data can be given on the reaction kinetics of the derivatization of the barbiturates, although it has been established by NMR spectroscopy that both NH groups react.

Chromatographic separations

Separations were carried out at room temperature with a fixed-wavelength detector at 254 nm. Fig. 2 shows the isocratic separation of the phthalimidomethyl fatty acid esters of a sunflower oil. The latter had been saponified with methanolic potassium hydroxide solution and extracted with acid.

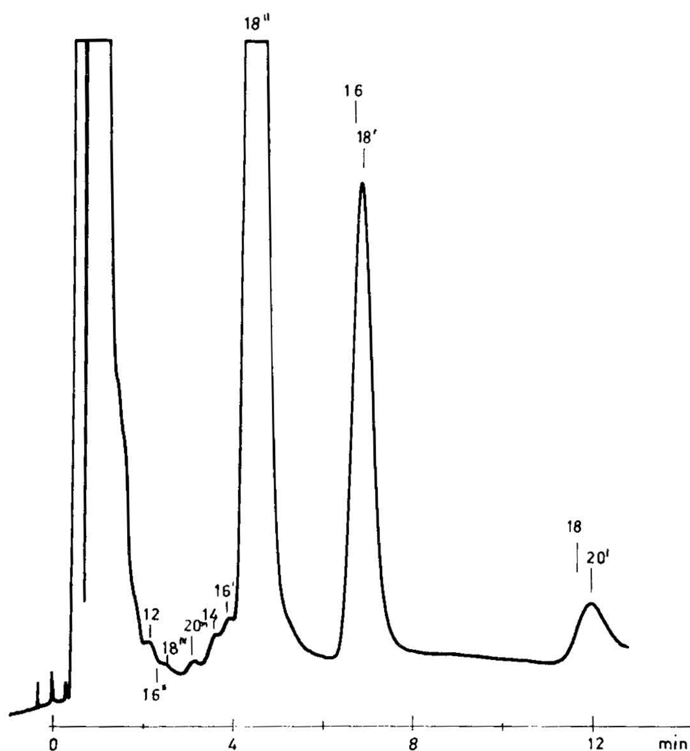


Fig. 2. Separation of the phthalimidomethyl esters of the fatty acids from sunflower oil. Column: HIBAR (Merck), C_8 LiChrosorb (7 μm), temperature 20°. Mobile phase, acetonitrile–water (9:1); flow-rate, 1.5 ml/min; detection, UV at 254 nm; range, 0.4. Peak designation: the numbers indicate the chain lengths of the given carboxylic acid and the superscripts indicate the number of double bonds.

Free fatty acid samples were derivatized by method B. The chromatographic identification of the individual saturated acids was facilitated by the fact that using the isocratic procedure the logarithms of the retention times yield a linear relationship when plotted against the number of carbon atoms of the acids. The same applies

to unsaturated homologous acids, although here the linear relationship has a different slope. The situation is closely analogous to the gas chromatographic identification of fatty acid methyl esters under isothermal conditions.

It can also be seen that the retention time decreases as the number of double bonds in a carbon chain increases, as for example in the C_{18} acids.

Highly unsaturated long-chain fatty acids, which can be determined only with great difficulty by gas chromatography owing to the very long retention times of the methyl esters, can be quickly and efficiently separated and assayed by HPLC using the UV-sensitive fatty acid esters and a mobile phase gradient programme.

As expected, the molar extinction coefficient (ε) is the same for all phthalimidomethyl monocarboxylic acid esters; dicarboxylic acid esters extinction coefficients that are twice as high. This phenomenon can be used conveniently for standardization in quantitative work[24].

Using the 4-nitrophthalimidomethyl esters and a wavelength of 254 nm, a lower limit of detection of 1–2 ng for acetic acid and of 10 ng for stearic acid are readily attainable.

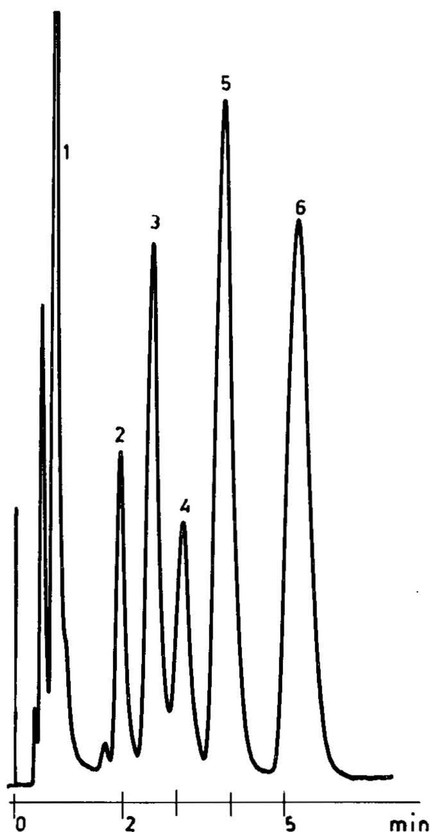


Fig. 3. Separation of the phthalimidomethyl diesters of the C_2–C_6 dicarboxylic acids. Column as in Fig. 2. Mobile phase, acetonitrile–water (3:2); flow-rate, 1.5 ml/min; detection, UV at 254 nm. Peak designation: 1 = 1-hydroxymethylphthalimide; 2 = ClMPI; 3 = malonic acid diester; 4 = succinic acid diester; 5 = glutaric acid diester; 6 = adipic acid diester.

As can be seen in Fig. 3, the dicarboxylic acid esters can also be separated successfully. Under the isocratic conditions described here the oxalic acid diester peak largely coincides with the reagent peak.

The reaction rate and degree of esterification of dicarboxylic acids with ClMPI or ClMNPI were not studied in detail, although they seemed to be comparable to those for the fatty acids.

The highly unstable α-ketocarboxylic acids can be derivatized successfully without decomposition. Fig. 4 illustrates the separation of three acids occurring in the Krebs citric acid cycle (α-ketoglutaric, succinic and fumaric acids) in the presence of excess of reagent, which had been derivatized by using method B or C. The reaction can also be carried out at room temperature.

It can therefore be applied successfully to the derivatization of thermolabile substances such as penicillin G, and the water-soluble sodium salts of the penicillins mostly employed in pharmaceutical dosage forms which can be used directly for the

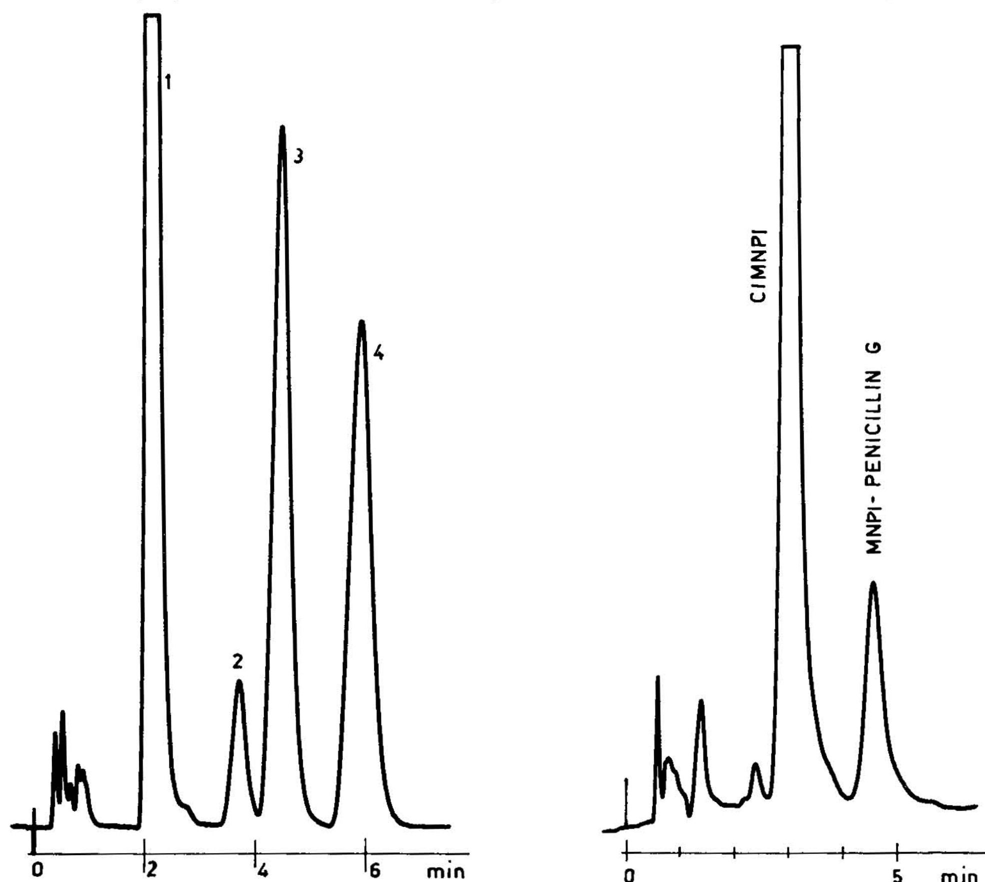


Fig. 4. Separation of Krebs cycle acids as 4-nitrophthalimidomethyl diesters. Separation system as in Fig. 2. Peak designation: 1 = ClMNPI; 2 = α-ketoglutaric acid diester; 3 = succinic acid diester; 4 = fumaric acid diester.

Fig. 5. Chromatogram of penicillin G 4-nitrophthalimidomethyl ester. Column and detection as in Fig. 2. Mobile phase, acetonitrile–water (1:1); flow-rate, 1.5 ml/min.

purpose. Fig. 5 shows an application of the principle. If necessary, the excess of the reagent can be destroyed by addition of triethylamine after derivatization. The product of this reaction precipitates.

As a final application, the isocratic separation of a barbiturate mixture derivatized with chloromethylphthalimide by method B is shown (Fig. 6). The reaction kinetics have not been studied in detail; yields of more than 90% were consistently achieved in trial preparations. The attachment of two chromophores greatly increases the molar extinction coefficients of most barbiturates, and at the same time their detectability at 254 nm improved to about 4 ng.

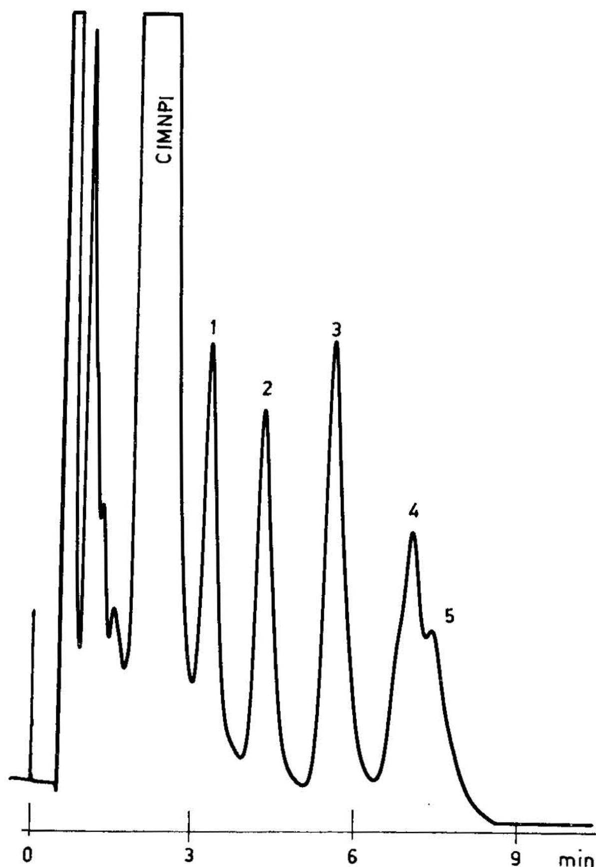


Fig. 6. Separation of a 4-nitrophthalimidomethyl-substituted barbiturate mixture. Separation system as in Fig. 3. Peak designation: 1 = methylphenobarbital derivative; 2 = phenobarbital derivative; 3 = cyclobarbital derivative; 4 = amobarbital derivative; 5 = secobarbital derivative.

The detection sensitivity of the barbital is enhanced by a factor of about 80 by the derivatization (at 254 nm). In this comparison it is assumed that the product peak height times retention time is comparable when isocratic elution is used.

The difference in the molar extinction coefficients at 254 nm between the derivatized barbital and barbital itself is about 10^3 (see Table I). With a signal-to-noise ratio of 5:1, 5 ng of derivatized barbital can be detected.

Recently, Clark and Chan[25] described the post-column ionization of barbiturates. The lower limit of detection of butalbital was 12.2 ng. In strongly alkaline solutions the barbiturates are ionized and a fully conjugated system of double bonds is formed. This results in a bathochromic shift of the UV absorption maximum and the absorbance is enhanced.

CONCLUSIONS

The advantage of the reagents described here over others such as 1-benzyl-3-*p*-talyltriazene[13,26] and 1-*p*-nitrobenzyl-3-*p*-talyltriazene[27] is that they are not limited to a particular functional group. From all of the examples described above it can be seen that the N-chloromethylphthalimides react relatively quickly and almost quantitatively with salts of NH and OH compounds and are thus very suitable UV-sensitive derivatization reagents. The derivatives possess good chromatographic properties and the limits of detection are in the nanogram range.

At present further studies are being undertaken to investigate the use of the reagents with important compounds with acidic functional groups, in particular compounds that occur in plants and other biological materials.

ACKNOWLEDGEMENT

We thank Mrs. M. Auf for her valuable cooperation.

REFERENCES

1 J. F. Lawrence and R. W. Frei, *Chemical Derivatization in Liquid Chromatography*, Elsevier, Amsterdam, Oxford, New York, 1976.
2 K. Blau and G. S. King, *Handbook of Derivatives for Chromatography*, Heyden & Son, London, 1977.
3 H. Böhme, R. Broese and F. Eiden, *Chem. Ber.*, 92 (1959) 1258.
4 H. Böhme and H. Schwartz, *Arch. Pharm. (Weinheim)*, 307 (1974) 775.
5 G. H. Nefkens, *Nature (London)*, 193 (1962) 974.
6 H. Böhme and H. H. Otto, *Arch. Pharm. (Weinheim)*, 300 (1967) 922.
7 H. Böhme and H. Schwartz, *Arch. Pharm. (Weinheim)*, 306 (1973) 684.
8 F. Knotz, *Sci. Pharm.*, 38 (1970) 227.
9 W. Wendelin and F. Knotz, *Monatsh. Chem.*, 103 (1972) 1632.
10 F. Knotz, *Sci. Pharm.*, 41 (1973) 123.
11 F. Knotz, *Sci. Pharm.*, 38 (1970) 26.
12 W. Wendelin, F. Knotz and H. W. Schramm, *Monatsh. Chem.*, 106 (1975) 159.
13 I. R. Politzer, G. W. Griffin, B. J. Dowty and J. L. Laseter, *Anal. Lett.*, 6 (1973) 539.
14 D. R. Knapp and S. Krueger, *Anal. Lett.*, 8 (1975) 603.
15 E. Grushka, H. D. Durst and E. J. Kikta, Jr., *J. Chromatogr.*, 112 (1975) 673.
16 W. Morozowich and S. L. Douglas, *Prostaglandins*, 10 (1975) 19.
17 R. F. Borch, *Anal. Chem.*, 47 (1975) 2437.
18 F. A. Fitzpatrick, *Anal. Chem.*, 48 (1976) 499.
19 P. T. Pei, W. C. Kossa and S. Ramachandran, *Lipids*, 11 (1976) 814.
20 H. D. Durst, M. Milano, E. Kikta, S. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
21 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.
22 E. H. Huntress and R. L. Shriner, *Org. Syn.*, 2 (1943) 429.
23 H. D. Durst, *Tetrahedron Lett.*, (1974) 2421.
24 F. Nachtmann, H. Spitzy and R. W. Frei, *Anal. Chem.*, 48 (1976) 1576.
25 C. R. Clark and P. L. Chan, *Anal. Chem.*, 50 (1978) 635.
26 E. H. White, A. A. Baum and D. E. Eitel, *Org. Syn.*, 48 (1968) 102.
27 *Regis Laboratory Notes*, Regis Chem. Co., Morton Grove, Ill., No. 16, August 1974.

Journal of Chromatography, 176 (1979) 65–73
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,892

LIQUID COLUMN CHROMATOGRAPHY OF NUCLEOSIDE MONOPHOSPHATES ON DEAE-SPHERON 1000

VRATISLAV SVOBODA and IMRICH KLEINMANN

Institute for Research, Production and Application of Radioisotopes, 17004 Prague 7 (Czechoslovakia)
(First received September 8th, 1978; revised manuscript received March 26th, 1979)

SUMMARY

The ion exchange capacity, radiation stability and particle distribution have been estimated for two fractions of the weak anion exchanger DEAE-Spheron 1000. Capacity factors are reported for eight nucleotides measured under various conditions in citrate and formate buffers. This type of sorbent may be used for the separation of deoxy- or ribonucleotides under mild conditions in ammonium formate buffer. No interferences from other substances which are usually found in samples of biological origin have been observed.

INTRODUCTION

Supports which have been used for the separation of nucleotides are either polystyrene-based strong anion exchangers such as Aminex A-27, A-28[1–3], chemically bonded strong anion exchangers on silica microparticles[4,5] or weak anion exchangers with a cellulose or dextran matrix such as DEAE-Sephadex[6,7]. To these well established column materials may be added[8,9] octadecyl-bonded silica.

However, all separation methods using these supports possess certain disadvantages. Thus the polystyrene-based anion exchangers, most commonly used, are chemically and thermally stable, but the aromatic rings within the matrix cause strong adsorption of the aromatic parts of nucleotides. This may be suppressed by increasing the temperature (usually to 70°), but this increases the rate of decomposition (hydrolysis) of nucleotides, especially in acidic buffers. On the other hand, the use of basic pH values may cause overlap of the elution volumes of nucleosides and nucleotides[3] and a two-step separation procedure may be needed. The silica-bonded ion exchangers increase the separation speed and may be used at room temperature, but their exchange capacity is usually low and some organic compounds, which may occur frequently in biological samples, may drastically decrease their efficiency[5]. The dextran-based ion exchangers are produced as relatively coarse particles and are easily compressible, therefore are not suitable for high-performance liquid chromatography. Finally, separation of nucleotides by reversed-phase chromatography on octadecyl bonded to silica has been described only recently[8,9] and its scope seems to be limited.

DEAE-Spheron is a weak anion exchanger produced by substitution of hydroxymethyl methacrylate–ethylenedimethacrylate copolymer beads[10] and has been used for the separation of a broad range of biologically important substances, *e.g.*, sugars[11] and mono-, di- and triphosphates of adenosine[12]. It is more hydrophilic than polystyrene-based anion exchangers, its rigidity in the swollen state is satisfactory and its capacity is higher than that of silica-based ion exchangers. Therefore it seemed worthwhile to try it for the separation of naturally occurring 5′-nucleotides.

EXPERIMENTAL

Apparatus

Glass columns of various lengths and inner diameter of 3.5 mm were used[13]. The eluents were stored in glass containers, degassed by heating to 70° and transported by pump MC 706.1 equipped with pumping head K or L (Mikrotechna, Prague, Czechoslovakia). A Bourdon-type manometer was used for pressure monitoring (Chirana, Prague, Czechoslovakia). A short precolumn (20 mm × 4 mm I.D.) filled with DEAE-Spheron 1000 was used for removal of particulate matter or impurities from the eluent. The eluent was monitored by a Uvicord III (LKB, Stockholm, Sweden) and by a conductivity detector described previously[14]. Signals were recorded by a four-channel Type 175 recorder (Kutesz, Budapest, Hungary).

Reagents

Adenosine 5′-monophosphate, sodium salt (AMP) (type II), 2′-deoxyguanosine 5′-monophosphate (dGMP), deoxycytidine 5′-monophosphate (dCMP), 2′-deoxyadenosine 5′-monophosphate (dAMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP) and thymidine 5′-monophosphate (TMP) were purchased from Sigma (Saint Louis, Mo., U.S.A.); all other reagents of p.a. quality were from Lachema (Brno, Czechoslovakia).

Sorbent

The sorbent DEAE-Spheron 1000 was a Lachema product and two fractions were available to us, having nominal particle diameters of 25–40 μm and of < 25 μm, respectively.

Particle size determination

Sorbent particles swollen in distilled water were photographed using an Amplival optical microscope (Carl Zeiss Jena, Jena, G.D.R.) with × 160 magnification optics, Exacta camera and a high-resolution ORWO NP 15 film. At the same setting, several frames were exposed to depict more than 500 particles, and a glass scale divided into 10-μm sections. From prints made under identical conditions, the diameters of 500 randomly selected particles were measured with a precision of *ca.* 0.1 mm (3–5 mm being the diameter of particles on the print). The measurements were statistically evaluated using a simple program written in BASIC (Varian 620 L computer). The diameter of the majority of particles ($> 96\%$) lies between 14 and 40 μm for the 25–40-μm fraction and between 8 and 29 μm for the $<$25-μm fraction. This distribution seems suitable for most preparative applications, but for some demanding separations the spread of particle sizes should be narrower. Statistical characteristics of both fractions are summarized in Table I.

TABLE I

DISTRIBUTION OF PARTICLE SIZES

Parameter	*Fraction 1*	*Fraction 2*
Nominal diameter (μm)	25–40	<25
Number average (μm), M_n	25.87	17.42
Weight average (μm), M_w	27.45	18.78
Standard deviation (μm), σ_n	6.41	5.02
Skew	0.69	0.18
Excess	0.46	0.01

Packing of columns

Typically, *ca.* 3 g of Spheron were stirred with ca. 50 ml of 1 *M* buffer at an appropriate pH and left to stand for *ca.* 1 h; then the supernatant was decanted and the whole procedure repeated five or six times. Finally, the wet sorbent was suspended in a concentrated solution of buffer and the slurry poured into a reservoir (100 mm × 18 mm diameter), with a column of the same solution attached to its lower end. Buffer solution (*ca.* 1 *M*) was fed into the reservoir at a pumping speed of *ca.* 26 ml/h. When the column was completely filled with sorbent, the reservoir was disconnected, the injector-head mounted on the column and the column washed with buffer of desired molarity and pH. This procedure guarantees attainment of high quality packing and is superior to all others we have tried (high-pressure, high-viscosity and low-density slurry packing).

RESULTS AND DISCUSSION

Measurement of interstitial volume

The determination of interstitial volume is important for the estimation of capacity factors. However, all of the nucleic bases, nucleosides and other compounds that we tried were to some degree retained on the column. The interstitial volume (*i.e.*, the total volume within the column accessible to the eluent) was best determined by conductimetric measurements of the slight fluctuations of buffer concentration in the effluent at the exit from the column. The column (250 × 3.5 mm), filled with DEAE-Spheron 1000 (25–40 μm), was operated at 50°. As elunt, 0.07 *M* ammonium formate buffer (pH 4.5) was used. When 50 μl of 0.5 *M* ammonium formate were injected, a conductivity peak (conductivity increase) at an elution volume of 1.58 ml was found. Under identical conditions, but 50 μl of water injected, an interstitial volume of 1.49 ml was found (eluent conductivity decrease peak). The larger volume found with the increased concentration of eluent, (*i.e.*, an increase of conductivity) is due to marked asymmetry of that peak, however, the difference in retention volumes is very small (*ca.* 6%). Changes in temperature (20–70°) pH (3–5), injected volume (10–50 μl) and concentration of injected formate (0.5–2 *M*) had no influence on the interstitial volume. An increase of eluent concentration (from 0.07 to 0.4 *M*) causes the interstitial volume to increase by 7%. A decrease in the mean particle size of the packing from 26 to 27 μm resulted in the interstitial volume increasing from 66 to 73% of the void volume of the column. When the length of the column packing was increased from 227 mm to 977 mm, there was no change in the relative interstitial volume.

Capacity of DEAE-Spheron 1000

Nominal ion exchange capacity values, supplied by the producer, were 1.5 ± 0.25 mequiv./g (dry sorbent) for the 25–40 μm fraction and 1.68 mequiv./g for the <25 μm fraction[15]. The ion exchange capacity of sorbent was determined by titration with perchloric acid. It is well known that the perchlorate ion has a high affinity for most anion exchangers. The dry sorbent was weighed and *ca.* 1 g was transferred to a beaker, rinsed five times with 30 ml 1 *M* NaOH, then (on a frit funnel) washed with 0.8 l of freshly boiled distilled water. It was then suspended in 20 ml water and titrated with 0.1 *M* $HClO_4$. The single additions of acid solution were *ca.* 1 ml; after every addition the solution was stirred until a steady pH was reached (*ca.* 15 min). The capacity so determined was 1.51 mequiv./g for the coarser fraction, in good agreement with the manufacturer's data.

The maximum amount of nucleotide which may be retained under typical conditions on the column packing was estimated from a break-through experiment. The column (61 × 3.5 mm) was equilibrated with 0.07 *M* ammonium formate and then a solution of AMP in water (1.62 mg/ml) was pumped through the column. As may be seen from Fig. 1, the inflection point in the concentration *vs.* volume curve corresponds to 13.2 mg AMP retained on the column, *i.e.*, to 0.25 mmol/g of dry sorbent. If the concentration of AMP solution was increased to 6.48 mg/ml, the inflection point corresponded to 0.67 mequiv./g of dry sorbent. Thus a fourfold increase of concentration corresponds to only a 2.7-fold increase of sorbed AMP. Obviously, if the sorption isotherm is no longer linear, even at relatively high concentration only a fraction of exchange sites is used.

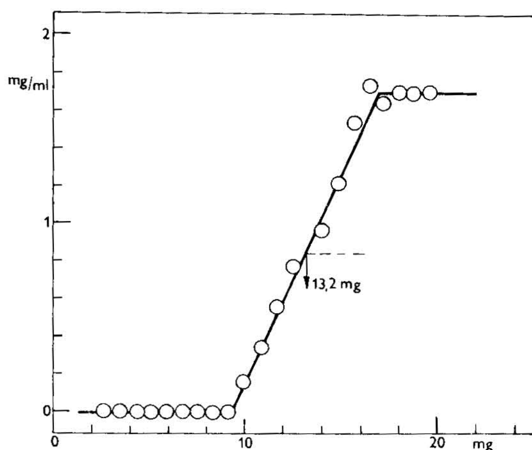


Fig. 1. Determination of capacity of DEAE-Spheron 1000 for AMP. Sorbent fraction: <25 μm. Eluent: solution of AMP in water, 1.62 mg/ml. Interestitial volume: 0.47 ml.

Citrate buffer

All measurements with citrate buffer were performed at 50° and pH 3.5. The concentration of buffer is given here as the molarity of sodium citrate; the citric acid used for the adjustment of acidity was not taken into account. So the nominal concentration corresponds approximately to the citrate-ion concentration. All com-

putations were performed using programs GRABAC and GRAPUR in BASIC (Varian 620 L computer). Approximately linear plots of log K_D *vs.* log c were obtained, with a slope of *ca.* −0.5 (Fig. 2). For monophosphates of ribonucleosides the critical pair is AMP–UMP, for the monophosphates of deoxyribonucleosides it is dAMP and dTMP. The separation of deoxy- and riboso-pairs derived from the same base is also poor. The influence of pH is more marked for the nucleotides derived from amino-substituted bases (CMP, dCMP, AMP, dAMP). Thus, on going from pH 3.5 to pH 4.5, the elution sequence of AMP and UMP (respectively dAMP–dUMP) is reversed. At the same time, the resolution of the deoxy- and riboso-pair improves; however, with a relatively short column it is still rather poor.

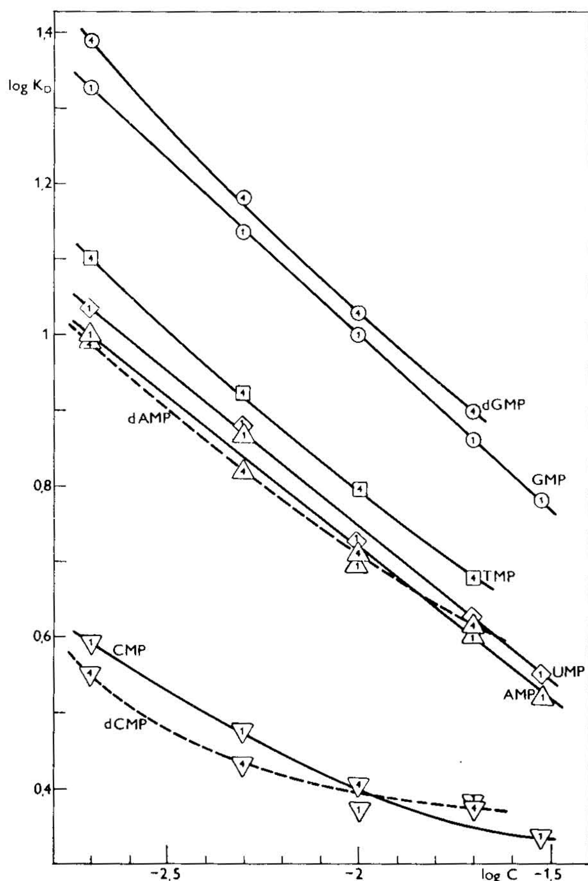


Fig. 2. Plot of capacity factors *versus* buffer concentration (logarithmic scales). Buffer: sodium citrate, pH 3.5. Column (250 × 3.5 mm) temperature: 50°. Pumping speed: 25 ml/h. Sorbent fraction: 25–40 μm.

Formate buffer

As with citrate buffer, the concentration of the formate buffer indicated in the graphs and tables corresponds to the molarity of ammonium formate; the pH was adjusted by addition of formic acid (pH < 6) or ammonia.

It is interesting to compare the results obtained with citrate and formate buffer at the same pH (Figs. 2 and 3). Formate ions have a much weaker affinity for the sorbent than citrate ions and in order to attain the same elution volume, the concentration of formate must be about 10 times greater. The plots of log K_D *vs.* log c are approximately linear, and the slope approaches the theoretical value of 1.0. The elution sequence in both buffers at pH 3.5 is the same for all eight nucleotides.

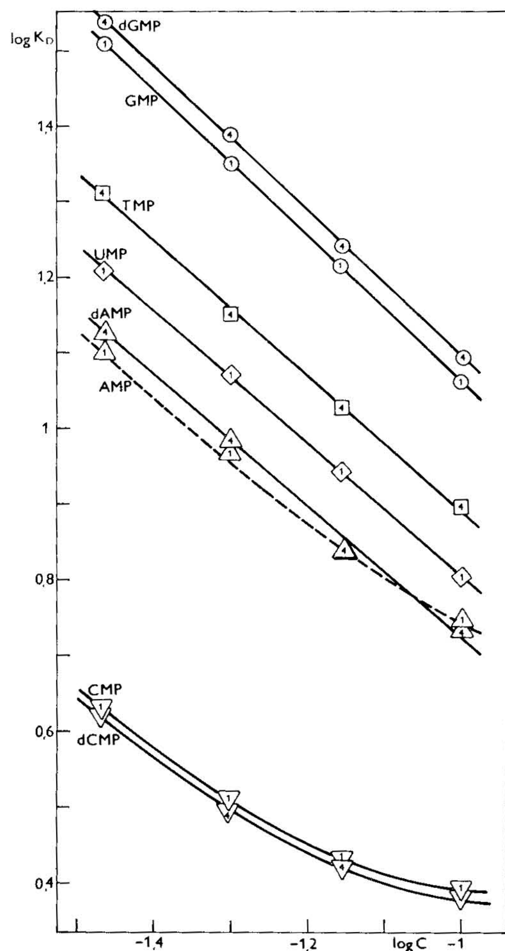


Fig. 3. Influence of concentration on capacity factors of nucleoside monophosphates. Eluent: ammonium formate buffer, pH 3.5. For other parameters see Fig. 2.

The influence of pH on capacity factor (in 0.07 *M* ammonium formate) is shown in Fig. 4. It can be seen that the optimum pH value for the separation of four ribonucleotides or deoxyribonucleotides is around pH 3 or pH 4.5. If all eight nucleotides are separated, the optimum pH value is 4.7. A change of elution sequence occurs at pH 3.7–4.0. At pH 7 the elution character is drastically changed: the differences between

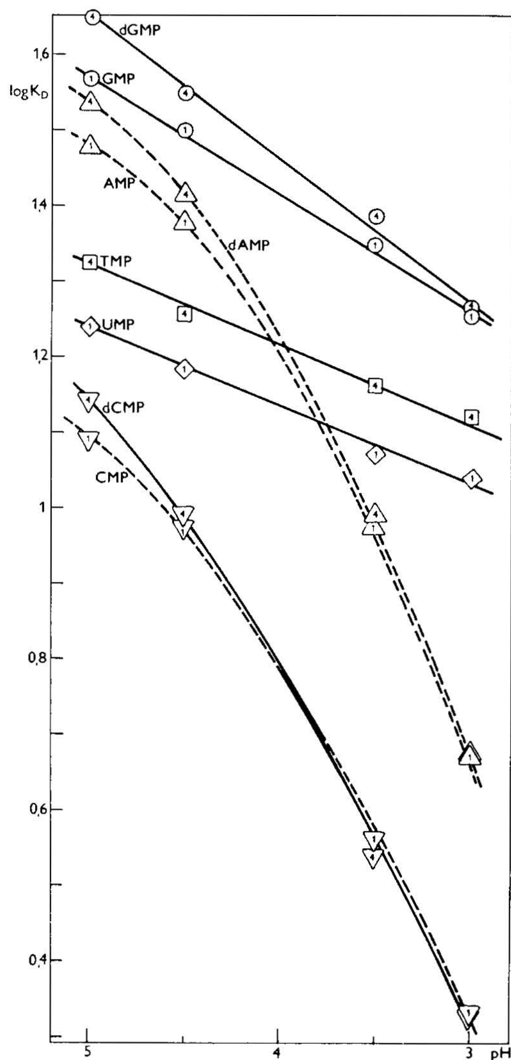


Fig. 4. Influence of pH on capacity factors of nucleoside monophosphates. Eluent: ammonium formate buffer, 0.07 *M*. For other parameters see Fig. 2.

purine or pyrimidine nucleotides are negligibly small, and the separation of AMP and GMP is practically impossible.

When the acidity of both the formate and citrate buffers is decreased, the peak widths increase. At higher eluent concentration and constant pH the peak width decreases.

Column temerature and flow-rate

The influence of temperature on the separation of nucleotides on DEAE-Spheron 1000 was followed quantitatively for the CMP–UMP pair. Results were collected for several consecutive days at temperatures of 30° and 50°. Using the

Student's criterion, it could be estimated (at the 99.9% confidence level) that the temperature markedly influences the resolution of this pair (see Table II). It is of interest that the elution volume of UMP decreases with increasing temperature, but that for CMP the retention is stronger at higher temperature. The column efficiency for UMP decreases with increasing temperature (99.9% confidence level), whereas the reverse is true for CMP. Finally, the higher resolution of this pair at lower temperature is mainly due to the change of retention volumes.

TABLE II

INFLUENCE OF TEMPERATURE ON SEPARATION OF CMP AND UMP

N = Number of theoretical plates, W = peak width, V_R = elution volume, R = resolution. Column, 250 × 3.5 mm; eluent, 0.07 M ammonium formate (pH 4.5); pumping speed, 9.8 ml/h. Sorbent: DEAE-Spheron 1000, 25–40 μm.

Compound	*T (°C)*	*V_R (ml)*	*W (ml)*	*N*	*R*	*No. of experiments*
CMP	30	5.185 ± 0.039	0.795 ± 0.014	679 ± 28	3.50	11
UMP	30	8.899 ± 0.091	1.329 ± 0.019	717 ± 18		
CMP	50	5.588 ± 0.020	0.799 ± 0.017	784 ± 32	2.64	8
UMP	50	8.370 ± 0.027	1.311 ± 0.026	652 ± 25		

If the linear speed of the eluent is decreased from 0.26 cm/sec to 0.014 cm/sec, the HETP decreases to about one fourth and resolution is increased *ca.* 1.8 times.

Radiation stability of DEAE-Spheron 1000

A short column filled with sorbent and buffer was subjected to irradiation by high-energy photons in a Gamacell radiation source (^{60}Co). The results are summarized in Table III. Even the highest absorbed dose (161 kiloGrey) did not markedly change the separation efficiency of this column. So, also in this respect the DEAE-Spheron 1000 compares favourably wtih polystyrene-based ion exchangers.

TABLE III

IRRADIATION OF CHROMATOGRAPHIC COLUNN

Column, 3.5 × 95 mm; pumping speed, 13.2 ml/h; DEAE-Spheron 1000, ~ 25 μm.

Dose absorbed (Gy)	*CMP*			*AMP*		
	K_D	*N*	*R*	*K_D*	*N*	*R*
0	1.74	290	—	9.59	387	5.64
$1.44 \cdot 10^4$	1.61	239	—	9.19	370	5.40
$4.032 \cdot 10^4$	1.65	234	—	9.55	376	5.57
$16.128 \cdot 10^4$	1.65	281	—	9.30	351	5.41

CONCLUSION

DEAE-Spheron 1000 may be used both for analytical and preparative separations of nucleotides from biological samples. The separation may be conducted under mild conditions (room temperature, pH 4.7). No interference from

substances commonly occurring in biological extracts (*e.g.*, Tris buffer) has been observed. The group separation of nucleosides and bases from the first eluting nucleotide, CMP, is good and therefore the one-step separation is satisfactory (see Fig. 5). The rigidity of the swollen gel seems to be higher than that of polystyrene-based ion-exchangers so that pressure gradients up to 100 kPa/cm may be used safely. The exchange capacity is similar to that of polystyrene-based ion exchangers and this may be used even for preparative separations. The peak widths under comparable conditions are much smaller on DEAE-Spheron gel than on polystyrene-based anion exchangers. Work is in progress in this laboratory to demonstrate the application of this gel for the separation of very complicated mixtures of nucleotides and similar substances.

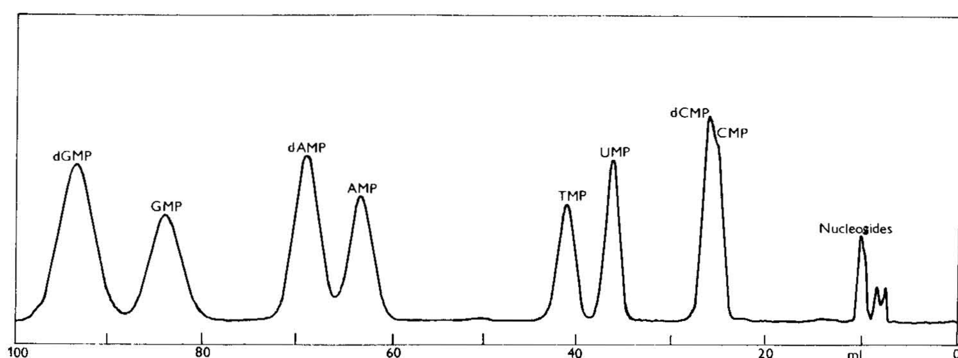


Fig. 5. Elution pattern of synthetic mixture of nucleosides and nucleoside monophosphates. Eluent: 0.07 *M* ammonium formate, pH 4.7. Column (980 × 3.5 mm) temperature, 50°; pressure, 1.2 MPa, Pumping speed: 26 ml/h. Sorbent: DEAE-Spheron 1000; fraction, 25–40 μm.

REFERENCES

1 R. P. Singhal, *Eur. J. Biochem.*, 43 (1974) 245.
2 J. X. Khym, *J. Chromatogr.*, 97 (1974) 277.
3 J. X. Khym, *J. Chromatogr.*, 124 (1976) 415.
4 P. A. Asmus, Ch. E. Low and M. Novotný, *J. Chromatogr.*, 119 (1976) 25.
5 R. A. Hartwick and P. R. Brown, *J. Chromatogr.*, 112 (1975) 651.
6 I. C. Caldwell, *J. Chromatogr.*, 44 (1969) 331.
7 T. Rosett, J. G. Smith, Jr., I. Matsuo, P. A. Bailey, D. B. Smith and S. Surakiat, *J. Chromatogr.*, 49 (1970) 308.
8 C. Horváth, W. Melander and I. Molnár, *Anal. Chem.*, 49 (1977) 142.
9 F. S. Anderson and R. C. Murphy, *J. Chromatogr.*, 121 (1976) 251.
10 O. Mikeš, P. Štrop and J. Čoupek, *J. Chromatogr.*, 153 (1978) 23.
11 Z. Chytilová, O. Mikeš, J. Farkaš, P. Štrop and P. Vrátný, *J. Chromatogr.*, 153 (1978) 37.
12 O. Mikeš, P. Štrop, J. Zbrožek and J. Čoupek, *J. Chromatogr.*, 119 (1976) 339.
13 V. Svoboda and I. Kleinmann, *J. Chromatogr.*, 148 (1978) 75.
14 V. Svoboda and J. Maršál, *J. Chromatogr.*, 148 (1978) 111.
15 M. Smrž, Lachema, Brno, personal communication.

Journal of Chromatography, 176 (1979) 75–87
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,845

QUANTITATION OF POLYMETHOXYLATED FLAVONES IN ORANGE JUICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. L. ROUSEFF and S. V. TING

Florida Department of Citrus, P.O. Box 1088, Lake Alfred, Fla. 33850 (U.S.A.)

(Received March 2nd, 1979)

SUMMARY

A quantitative high-performance liquid chromatographic (HPLC) procedure for the determination of the five major polymethoxylated flavones (PMFs) in orange juice has been developed. It employs a unique ternary solvent system with coupled UV–fluorescence detection. The dual detectors were employed to determine the presence of interfering substances and served as a cross check on quantitation. Stop flow UV and fluorescence scanning was used to identify peaks and determine the presence of impurities. Although all five citrus PMFs fluoresce, some HPLC fluorescence peaks were too small to be of much practical use. All five citrus PMFs could be quantitated satisfactorily with the fixed wavelength UV (313 nm) detector.

The HPLC procedure has been used to evaluate each step in the preparation. The optimum extracting solvent was selected and one time consuming step was eliminated, as it was found to be unnecessary. HPLC values for nobiletin and sinensetin are in good agreement with the thin-layer chromatographic (TLC) values in the literature. HPLC values for the other three flavones were considerably lower than those reported in the literature. The HPLC procedure is considerably faster than the TLC procedure with equal or superior precision and accuracy.

INTRODUCTION

While flavones are widely distributed in the plant kingdom, various citrus species are notable for their relatively large concentrations of highly substituted polymethoxylated flavones (PMFs). The highest concentrations of PMFs are found in citrus peel with much lower amounts found in the juice[1]. Certain citrus species contain characteristic PMF concentration patterns. Thus, relative concentrations of PMFs can be used to detect qualitatively the presence of one species in the juice of another[2]. Methoxylated flavones were originally suspected to contribute to occasional citrus juice bitterness[3,4], but were later reported to exist below taste threshold levels in orange juice[1].

Methoxylated flavones also produce important physiological responses in the higher animals. Kupchan *et al.*[5] reported tetramethylscutellarein to be a cytotoxic agent toward different strains of carcinoma cells. Other citrus PMFs have been re-

ported by Robbins[6] in *in vitro* experiments to regulate erythrocyte aggregation and concentration in human blood. He suggested possible dietary control of the high blood viscosity syndrome using foods such as citrus as a source of PMFs.

Separation and quantitation of these compounds has until now been done via the TLC–spectrophotometric procedure developed by Swift[3] and later modified by Veldhuis *et al.*[1]. The method is extremely lengthy, involving several manipulative steps which are possible sources of inaccuracy. Therefore, the goal of this study was to develop a more rapid method to accurately determine PMF levels in orange juice. These values could then be used to establish dietary PMF ingestion levels from orange juice for clinical studies.

EXPERIMENTAL

Reagents and standards

All chromatographic and extraction solvents were high purity UV grade purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Mallinckrodt (St. Louis, Mo., U.S.A.) Nanograde benzene was used for the benzene extractions. All water used in the mobile phase was deionized, distilled and filtered with a 0.22-μm Millipore (Bedford, Mass., U.S.A.) filter before use.

PMF standards were prepared by Lyle J. Swift, formerly of the U.S. Fruit and Vegetable Products Laboratory, Winter Haven, Fla., U.S.A. and were supplied by R. C. Robbins of the Department of Food Science and Human Nutrition at the University of Florida, Gainesville, Fla., U.S.A. Chromatographic analysis indicated that the standards could be used without further purification. Standard PMF solutions were prepared by adding the appropriate amount of solid flavone to a small volumetric flask and diluting to volume with absolute ethanol. The flasks were sonicated to facilitate dissolution.

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump and U6K injector were used as the solvent-sample delivery system in isocratic experiments, a Perkin-Elmer (Norwalk, Conn., U.S.A.) Series 3 pump with programmable gradient was used in all gradient studies. A Tracor (Austin, Texas, U.S.A.) Model 970A variable wavelength UV detector equipped with the wavelength scanning accessory was used to determine the spectra of trapped peaks in stop flow experiments. Two 8-μl cells were used as reference and sample cell, respectively. A Waters Assoc. Model 440 UV detector with a 313-nm filter kit was used as the general UV absorbance monitor. Both the general fluorescence monitoring and stop flow emission and excitation spectrum experiments were done with a Perkin-Elmer Model 204A fluorescence detector equipped with a square 20-μl flow cell. Individual PMF concentrations were determined from their integrated peak areas using the external standard method. A Spectra-Physics (Santa Clara, Calif., U.S.A.) Model 4000 integrator-printer plotter was used .

Chromatographic conditions

The PMFs were separated isocratically in the reversed-phase mode using a DuPont Zorbax C_8 column, 25 cm $\times$ 4.6 mm I.D. Isocratic mobile phase composi-

tion consisted of tetrahydrofuran (THF)–acetonitrile–water (22:6:72). Mobile phase solvents were degassed with vacuum and sonication before use. The flow-rate was 1.5 ml/min with a column head pressure of approximately 2200 p.s.i. The column was operated at ambient temperature, usually 22–25°.

Gradient mobile phase composition consisted of THF–water (22:78) for the weak solvent and THF–acetonitrile–water (22:40:38) for the stronger solvent. The concentration of THF was held constant throughout the solvent program to minimize baseline shifts. Initially, 15% of the strong solvent was mixed with the weak solvent. Thus, the mobile phase consisted of THF–acetonitrile–water (22:6:72). This composition was maintained for 10 min. Then the concentration of the strong solvent was increased linearly to 50% in 1 min. This composition (THF–acetonitrile–water, 22:20:58) was held constant for 7 min. Finally, the system was purged at 100% strong solvent for 5 min.

Each flavone peak was trapped in the detector cell as it reached its maximum peak height. Its UV absorption or fluorescence spectrum was obtained by scanning the wavelength region 390–200 nm and 220–550 nm, respectively. Then the flow was resumed.

Resolution of incompletely resolved chromatographic peaks was estimated using the method of Snyder[7].

Sample preparation

Single strength or reconstituted orange concentrate juices, to which Celite was added, were used and filtered with suction through Whatman No. 1 filter paper. Five grams of sodium hydroxide were rapidly dissolved in each 100 ml of juice that was to receive the alkaline treatment and allowed to stand for 30 min. All juices were extracted three times with 25 ml of benzene. The benzene extracts were combined and evaporated to dryness using a rotary evaporator with aspirator vacuum at 40°. Each residue was redissolved in 2.00 ml of absolute ethanol, filtered through a 1.2-μm filter and stored (refrigerated) in a septum sealed vial until injection.

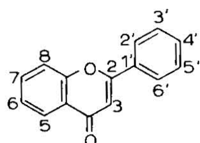
Identification of peaks

Chromatographic peaks were identified in several ways. Retention times of unknown peaks were compared with retention times of authentic compounds. Sample extracts were fortified with small amounts of standard material one at a time and re-chromatographed to see if the peak of interest increased in height. Spiked peaks were also checked for peak symmetry to determine if more than a single component might be present. Stop flow UV and fluorescence scans were obtained for each of the five major PMFs and compared to standards.

RESULTS AND DISCUSSION

Chromatographic separation

All of the PMFs have the basic flavone structure shown below. Since they



Sinensetin	5,6,7,3',4'-pentamethoxyflavone
Nobiletin	5,6,7,8,3',4'-hexamethoxyfllavone
Heptamethoxyflavone	3,5,6,7,8,3',4'-heptamethoxyflavone
Tetramethylscutellarein	5,6,7,4'-tetramethoxyflavone
Tangeretin	5,6,7,8,4'-pentamethoxyflavone

differ only in the position and number of methoxy groups, differences in polarity and solubility are subtle. Owing to the aromatic nature of these compounds, a C_{18} column was used for the initial separation. A C_8 column was later found to give slightly better separation and was used from that point on.

The choice of mobile phase constituents and proportions is critical to the effective separation of these flavones. Water–methanol or water–acetonitrile in various proportions could not adequately separate nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) from 3,5,6,7,8,3′,4′-heptamethoxyflavone. Resolution between these two compounds was never greater than 0.5 using 10-μm column packing material. If 5-μm column packing material was used, the resolution increased to approximately 0.8. Water–THF was the only solvent system which adequately resolved these two compounds. Resolution with water–THF (75:25) is approximately 1.3. It was found

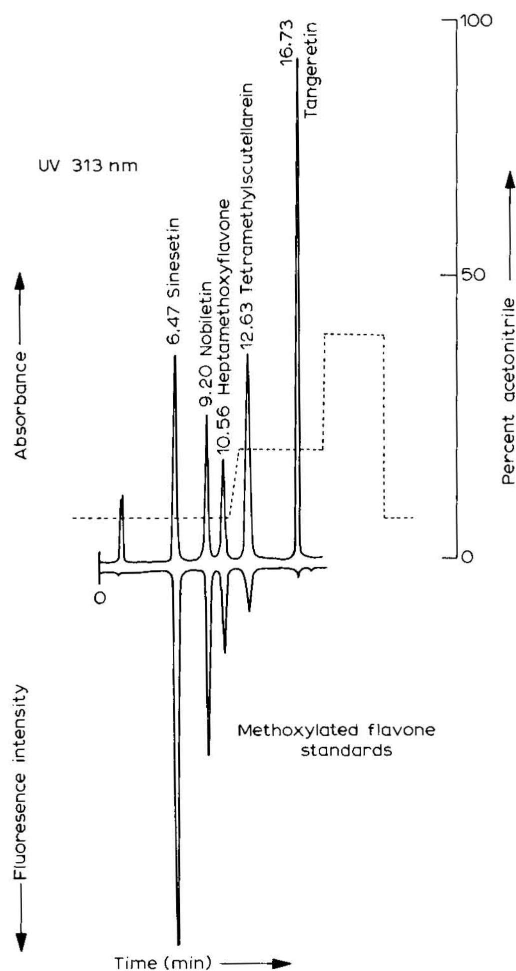


Fig. 1. Liquid chromatograms from a 5-μl injection of 100 ppm each of the five major polymethoxylated flavones in citrus. Upper chromatogram: UV absorbance (313 nm) at 0.1 a.u.f.s. Lower chromatogram: fluorescence determined with Fluoromonitor (ex. 360 nm, em. $>$415 nm) at 30×. Chromatographic conditions see text.

that by adding a small amount of acetonitrile and decreasing the THF concentration, the peaks became narrower and heptamethoxyflavone shifted further away from nobiletin, thus further increasing the resolution. The optimum ternary solvent mixture of water–THF–acetonitrile was 72:22:6, which placed heptamethoxyflavone equally distant between nobiletin and tetramethyscutellarein. This solvent system was satisfactory for all juice samples tested except those that had been treated with NaOH. The sodium hydroxide-treated samples required a solvent gradient to elute compounds formed as a result of the alkali treatment in a reasonable length of time. An example of the gradient separation is illustrated in Fig. 1 for the five standard PMFs. Since the concentration of THF remains constant, the solvent program is illustrated in terms of percent acetonitrile only. It is superimposed on the UV chromatogram in Fig. 1.

Detector response

It should be noted from Fig. 1 that UV absorbance at 313 nm more effectively detects all five major citrus PMFs than fluorescence. While the fluorescence detector is extremely sensitive to sinensetin, it is much less sensitive to the other flavones. The spectral characteristics of these compounds are given in Table I.

TABLE I

PMF SPECTRAL CHARACTERISTICS

Compound	*Fluorescence maxima (nm)**		*UV absorbance maxima (nm)*		
	Excitation	*Emission*	*Found**	*Literature***	*Log ε***
Sinensetin	346 (47)***	447 (71)	328	329	4.43
Nobiletin	352 (47)	462 (82)	331	333	4.45
Heptamethoxyflavone	330 (66)	466 (70)	342	341	4.38
Tetramethylscutellarein	331 (47)	492 (98)	319	318	4.58
Tangeretin	335 (53)	415 (71)	320	323	4.49

* THF–acetonitrile–water (22:6:72).
** Data from ref. 4; solvent: absolute ethanol.
*** Peak width at half height in parentheses.

Swift[4] reported the UV maxima for these compounds in absolute ethanol. To determine the authenticity of the standards used in these experiments, the individual standards were dissolved in absolute ethanol and their UV maxima were determined. There was excellent agreement with the values reported by Swift[4]. Even values obtained in the ternary solvent mobile phase using the stop flow technique differed not more than 3 nm from Swift's values. Since the extinction coefficients do not vary greatly, the relative sensitivities of these compounds can be explained by the proximity of their UV maxima to that of the 313-nm source.

As a means of qualitative identification, fluorescence excitation and emission maxima were determined for the five standards using the stop flow method. To further characterize these flavones their emission and excitation peak widths at half heights were also determined. Miller and Faulkner[8] have shown that the combination of such spectral features will allow excellent qualitative identification of compounds from liquid chromatography effluents.

Fluorescence excitation maxima for all five citrus PMFs are within 22 nm of

one another while emission maxima occur over a 77 nm range. Using a filter fluorometer with a narrow pass excitation filter (360 nm maximum and 55 nm band pass width) and a sharp cut-off emission filter (415 nm) it should be possible to detect all of the five flavones with approximately equal sensitivity. However, judging from their fluorescence peak heights in Fig. 1, it can be seen that some PMFs appear to have stronger fluorescence than others. To determine if this uneven response was an instrumental artifact, a variable wavelength fluorescence detector was used in place of the filter fluorometer. Excitation and emission wavelengths were set to optimize fluorescence for the smallest chromatographic peak, tangeretin. However, relative peak heights did not change. Thus, tangeretin's small fluorescence peak is not due to fluorescence undetected because of filter choice.

It has been reported[9,10] that the presence of oxygen will reduce fluorescence intensity. To determine if oxygen was causing the reduced fluorescence of the other four flavones, a chromatogram of the five standards was obtained in the usual manner. Helium was bubbled through the solvent saturating it and the chromatogram repeated. There was no change in peak heights. Thus, oxygen is not the cause of the reduced fluorescence intensities of the four methoxylated flavones.

Several factors could be responsible for these widely varying fluorescence intensities. Apparently there are sizable differences in the quantum efficiencies between these flavones. In addition, it was observed during the stop flow scans that some fluorescence peaks increased in size when they were trapped in the cell. Tangeretin was tripled in size within 10 min. This suggests that tangeretin exhibits some delayed fluorescence possibly due to phosphorescence, which is unusual for a solution at room temperature. Therefore, in the case of tangeretin, the small fluorescence peak size is due to delayed fluorescence and limited detector residence time under constant flow conditions. Tangeretin may also have a lower quantum efficiency than sinensetin.

Therefore, the fluorescence detector is not well suited as a general detector for these PMFs. While it is extremely sensitive to sinensetin, it is extremely insensitive to tangeretin under constant flow conditions.

Sample preparation

As a means of sample clean up, Swift[4] added NaOH until a 5% solution was achieved and allowed the solution to stand for 2 h before extraction. Veldhuis *et al.*[1] modified the procedure by shortening the alkaline reaction time to 30 min.

To determine the effectiveness of the NaOH treatment one portion of an orange juice (OJ) sample was treated with NaOH in the manner described by Veldhuis *et al.*[1] before extraction, while the other was immediately extracted. The resulting chromatograms are illustrated in Figs. 2 and 3. Both UV absorbance (313 nm) and fluorescence were monitored.

The purpose of treating the sample with NaOH was to obtain an extract with fewer interfering compounds. However, in comparing the UV chromatograms of the NaOH treated sample with that of the untreated sample, it becomes apparent that while a few peaks have been eliminated, several new peaks are observed. Specifically, two peaks occur near the sinensetin peak (t_R = 6.5 min). Usually one of these compounds will coalesce with sinensetin (see Fig. 3) causing a positive error in the integration of this peak. There are also two large peaks (t_R = 20.85 and 22.51 min) that elute after tangeretin, which are not present in the untreated juice. Apart from ex-

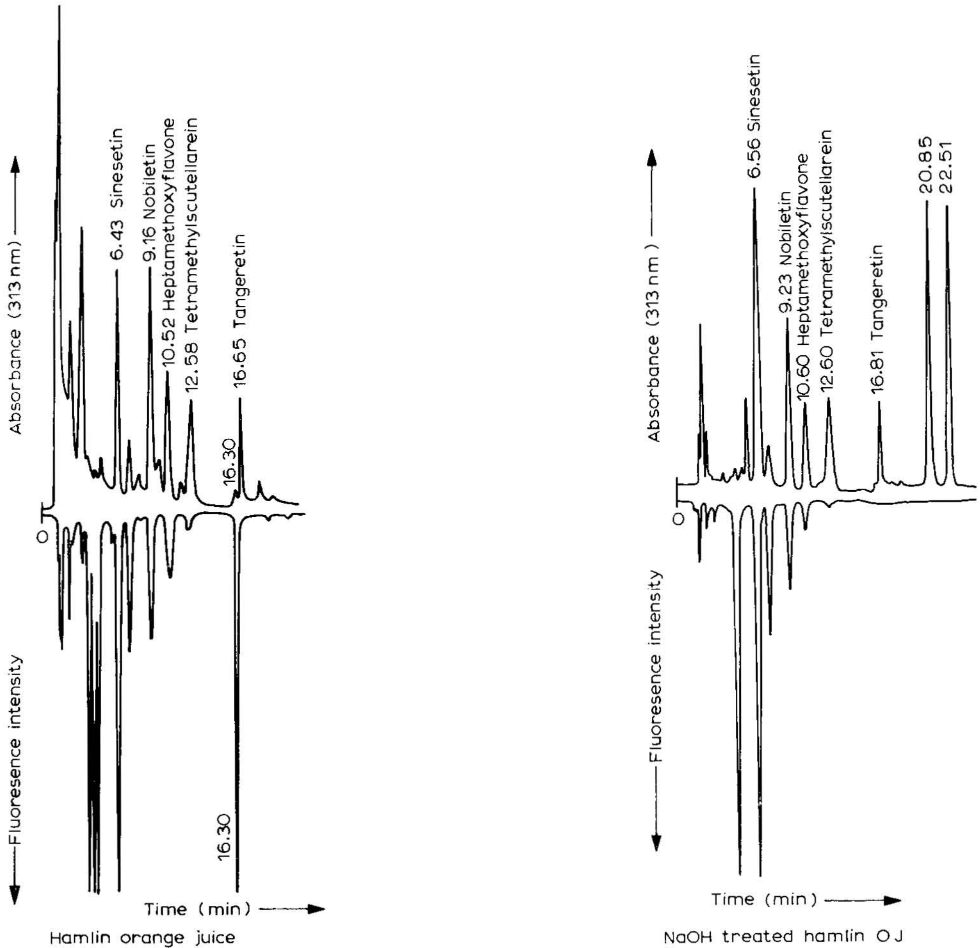


Fig. 2. Liquid chromatograms from a 20-μl injection of an orange juice extract. Chromatographic conditions and detectors as in Fig. 1.

Fig. 3. Liquid chromatograms from a 20-μl injection of an extract from an orange juice that has been treated with NaOH for 30 min before extraction. Chromatographic conditions, and detectors as in Fig. 1.

tending analysis time, these peaks present no problem in the gradient mode. However, if these samples were analyzed isocratically, these peaks would appear at much longer retention times. Usually they appear in subsequent chromatograms as low broad peaks that often interfere with the peaks of interest. On the other hand, the UV chromatogram of the juice sample that was not treated with NaOH contains two small potentially interfering peaks (one preceding tetramethylscutellarein and the other just following nobiletin) that are absent or reduced in the NaOH-treated sample. However, these potentially interfering peaks account for less than 10% of the height of the peaks of interest so any error caused by the inclusion of these peaks with the peaks of interest would be minimal.

There are some interesting qualitative differences between the NaOH-treated and untreated juice chromatograms obtained with a fluorescence detector. The NaOH-treated sample gave a much simpler chromatogram. Each of the five major citrus PMF peaks were well separated with good peak shape. The fluorescence peak at 7.2 min is probably due to the same substance Swift[4] observed as a blue-white band between nobiletin and sinensetin in his TLC studies. Judging from the trends in retention time and methoxylated substitution pattern observed in this study this compound is probably 3,5,6,7,3′,4′-hexamethoxyflavone reported by Tatum and Berry[11] in citrus peel. However, no fluorescence characteristics have been found in the literature and no authentic sample was available to test the validity of this tentative identification.

In the chromatogram of the untreated juice sample there were several major extraneous peaks that preceded the sinensetin peak and a very intense unidentified peak which occurred at 16.3 min (tangeretin occurs at 16.7 min). As the retention time of this latter peak was so close to that of tangeretin both fluorescence maxima were determined (340 nm and 447 nm) and compared to that of tangeretin. They are sufficiently different to indicate that the peak is due to something other than tangeretin. Judging from the lack of UV absorbance at the same retention time as the large fluorescence peak, this compound is apparently a relatively weak UV absorber and does not interfere with the UV peak of tangeretin (313 nm).

Peak shape studies suggest that there is an additional compound underneath the heptamethoxyflavone peak. Stop flow spectral scans gave falsely high values for heptamethoxyflavone. The tetramethylscutellarein peak (12.6 min) is also somewhat distorted but its intensity is so low it was not possible to determine from peak shape studies or stop flow spectral scans whether another compound is definitely present.

To determine the effects of NaOH treatment on individual and total PMF content, portions from the same lot of fresh squeezed Hamlin OJ were given two different NaOH treatments while a third portion received no treatment. The NaOH-treated samples differed only in the length of alkaline reaction time before extraction. Table II contains the concentrations of individual PMF's as determined from UV and fluorescence detectors. Integration of both fluorescence and UV chromatographic peaks should give complementary values.

Of the five major citrus PMFs, tangeretin was found in the lowest concentra-

TABLE II

EFFECT OF NaOH TREATMENT ON METHOXYLATED FLAVONES IN ORANGE JUICE

Juice concentration given in ppm; All samples were extracted 3 times with benzene.

Treatment	*Sinensetin*		*Nobiletin*		*Heptamethoxyflavone*		*Tetramethylscutellarein*		*Tangeretin*		*Total*	
	UV	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*
None	0.86	0.92	1.18	1.12	0.68	1.10	0.36	0.40	0.07	—	3.15	3.54**
NaOH (30 min)	1.04	0.84	0.90	1.00	0.66	0.74	0.30	0.32	0.06	—	2.96**	2.90
NaOH (60 min)	1.08	0.72	0.78	0.88	0.64	0.70	0.28	0.30	0.06	—	2.84**	2.60

* Contains falsely high value from heptamethoxyflavone.

** Contains falsely high value from sinesetin.

tion in OJ. There was no significant difference between NaOH-treated and untreated samples. Since the fluorescence detector was too insensitive to determine tangeretin, no comparison could be made with the UV values. There was substantial agreement between UV and fluorescence values for tetramethylscutellarein. The fluorescence values for this flavone were slightly higher in the untreated juice, possibly due to a fluorescent impurity. However, the UV values also show a slight decrease in tetramethylscutellarein concentration due to NaOH treatment and further decrease with increased alkaline reaction time. However, these differences are so slight that it is questionable whether they are significant.

Heptamethoxyflavone values suggest that this PMF is not affected by NaOH treatment. While the fluorescence value of the untreated juice is substantially higher than the alkaline treated samples, the corresponding UV values are very similar. The discrepancy between the UV and fluorescence values can be resolved as this peak was found to contain a fluorescent impurity.

Fluorescence sinensetin values decrease when the juice sample is treated with NaOH and continues to decrease as the alkaline reaction time is increased. Thus, it appears that sinensetin is degraded by NaOH. Sinensetin UV values are actually higher in the NaOH-treated samples than the untreated samples. This is probably due to an unresolved impurity which is formed as a result of the alkaline treatment and has been discussed earlier. Thus, the UV values do not reflect true sinensetin content.

The major PMF found in OJ is nobiletin. It also appears to be degraded by NaOH as evidenced by the drop in its concentration when treated with NaOH. Its concentration continues to drop the longer it remains in alkaline solution before extraction. Since both UV and fluorescence values are in excellent agreement, the possibility of this trend being due to some artifact is remote.

It can be seen from Table II that the total PMF content decreases when OJ samples are treated with alkali and that the decrease is proportional to the length of alkaline reaction time before extraction. While the UV total PMF concentration of the 1 h treated sample is only 10% lower than that of the untreated sample, it should be remembered that the alkaline-treated samples also contain an interfering substance in the sinensetin peak giving sinensetin and total PMF values which are falsely high. Comparing fluorescence values it appears that 27% of the PMFs from the untreated juice are lost when the sample is allowed to react with alkali for 1 h. This again is not correct as the total fluorescence values contain falsely high heptamethoxyflavone values. A reasonable estimation of the magnitude of the effect of NaOH treatment may be obtained by comparing the values of total UV PMF content of the untreated sample with total fluorescence PMF content of the alkali treated samples. Thus, there is approximately a 9% loss of total PMF content with the 30-min NaOH treatment and an 18% loss for the 1-h treatment.

Hydroxylated flavones are known to react under alkaline conditions. Seshadri[12] has used color changes in alkaline buffers to determine the number and positions of hydroxyl groups in flavones. Therefore, it should not be surprising that the methoxylated flavones should also react in alkali, but at a much slower rate.

Extraction efficiencies

Since Veldhuis *et al.*[1] used benzene to extract PMFs from OJ while Ting *et al.*[2] used chloroform, these and other solvents were evaluated for their ability to extract

PMFs from juice. All samples were taken from the same lot of fresh Hamlin OJ. Table III illustrates the results of this study. It can be seen that while chloroform is more convenient to work with and does not present the safety hazard benzene does, it is only about half as effective in extracting PMFs from OJ. When juice is treated with NaOH the chloroform extract exhibits the same problems as previously discussed for the NaOH-treated benzene extraction. Since there are no qualitative advantages in terms of types or numbers of compounds co-extracted with the flavones and in light of the severe quantitative short-comings ,chloroform is not the solvent of choice when total citrus PMF extraction is attempted.

TABLE III

SOLVENT EXTRACTION EFFICIENCIES

Solvent	*Sinensetin*		*Nobiletin*		*Heptamethoxy-flavone*		*Tetramethyl-scutellarein*		*Tangeretin*		*Total*	
	UV	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*	*UV*	*Fluor.*
Benzene	0.64	0.66	1.04	1.01	0.56	1.66	0.26	0.62	0.08	—	2.58	3.95
Chloroform	0.51	0.34	0.61	0.53	0.42	0.86	0.15	0.32	0.04	—	1.64	2.05
MIBK	0.55	0.54	1.10	0.92	0.74	1.40	0.29	0.44	0.06	—	2.74	3.30
Light petroleum	0.08	0.12	0.34	0.39	0.38	0.54	0.12	0.20	0.04	—	0.96	1.25

In terms of total PMFs found, methyl isobutyl ketone (MIBK) is more effective than chloroform and only slightly less effective than benzene. It is interesting to note that MIBK appears to be even more effective than benzene (as evidenced by UV concentration values) in extracting heptamethoxyflavone. However, it also extracts a very large amount of relatively polar compounds which greatly complicate the early portion of the chromatogram and possibly interfere with sinensetin. Total PMFs extracted with light petroleum were very low, indicating that this solvent is also less desirable than others as an extracting solvent. Toluene and hexane were also evaluated. As might be expected, toluene gave almost the same PMF values as benzene, and hexane gave values very similar to those of light petroleum.

Recovery studies

Base level concentrations of the five citrus PMFs were determined on a single lot of single strength Hamlin OJ. Known amounts of each of the five PMFs were added at two levels to a different portion of the same lot of OJ and analyzed. The results are shown in Table IV. Excellent recoveries were obtained for all of the PMFs, except heptamethoxyflavone which was inexplicably (and repeatably) low. Swift[4] reported excellent recoveries for all five citrus PMFs. However, his values were based upon amounts of standards recovered from silica gel plates only. No recovery test from known amounts of standards added to a sample were reported. No other recovery studies have been found in the literature.

Precision

The overall precision of this method is illustrated in Table IV. To determine

TABLE IV

FLAVONE RECOVERY STUDY

UV detection at 313 nm.

	Mean amount found (ng) *	*Amount added (ng)*		*Mean amount recovered*		*Recovered (%)*	
Sinensetin	344 (50)**	200	300	236	310	118	103
Nobiletin	474 (24)	300	450	284	422	95	94
Heptamethoxyflavone	210 (20)	100	150	62	104	62	69
Tetramethylscutellarein	100 (4)	100	150	92	132	92	88
Tangeretin	82 (8)	76	150	78	170	103	113

* 10-μl injection of juice extract.
** Standard deviation in parentheses, $n = 10$.

base level concentrations of citrus PMFs, five samples of the same juice were extracted and analyzed in duplicate. It can be seen from the standard deviation values that the overall precision is excellent for a natural product analysis at the ppm level. As might be expected, sinensetin has the lowest level of precision as evidenced by its 15% relative standard deviation (RSD). Heptamethoxyflavone has a relatively high RSD (9.5%) because it is relatively small and close to nobiletin. Since this study was performed isocratically, the tangeretin peak came out as a low broad peak. Thus, the precision for this peak (RSD = 9.8%) could probably be improved by optimizing integration parameters for a peak of this shape. Nobiletin and tetramethylscutellarein had RSDs of 5.1 and 4.0%, respectively.

Detection limits and linearity of response

UV detection limits for the fixed-wavelength (313 nm) detector range from 2 ng for tetramethylscutellarein and sinensetin to 8 ng for heptamethoxyflavone. Detection limits were considerably higher with the variable wavelength UV detector. (Detection limits were defined as that amount that would give a peak height of twice the peak to peak noise level.)

Fluorescence detection limits were not established because of the extremely unequal response to the PMFs in this study.

Linearity ranges between PMF concentrations and peak areas were established for the fixed wavelength UV detector. The response of all five flavones was found to be linear between 50 ng and 1.0×10^4 ng. All sample injections were well within this range. A linear least squares fit of this data indicated that sinensetin had the best correlation coefficient ($r = 0.999$) while tetramethylscutellarein had the lowest ($r = 0.991$).

PMF values in orange juice

HPLC flavone values for OJ are considerably lower than the corresponding TLC values reported by Veldhuis *et al.*[1]. A comparison between HPLC flavone values and those reported by Veldhuis *et al.* is shown in Table V. The HPLC values are from a typical frozen concentrated orange juice (FCOJ). All juices were reconstituted to 12° Brix. Total HPLC PMFs were only about half that found in the TLC procedure. Of the five flavones, only nobiletin values were comparable. Sinensetin values were

slightly lower while the other three flavones were considerably lower. Since the TLC procedure employed a NaOH treatment in sample preparation (which appears to reduce the concentrations of some PMFs, see Table II), it is surprising to find PMF values higher than those of the HPLC procedure which omits this step. Seasonal or varietal differences can not account for the magnitude of difference between these two sets of data.

TABLE V

COMPARISON OF PMF VALUES IN FCOJ RECONSTITUTED TO 12° BRIX

Method	*PMF concentration (ppm)*					
	Sinensetin	*Nobiletin*	*Heptamethoxyflavone*	*Tetramethylscutellarein*	*Tangeretin*	*Total*
HPLC	0.73	1.00	0.57	0.27	0.13	2.7
TLC*						
Average	0.90	1.31	0.81	0.51	0.45	4.24
Range	0.7–2.05	0.8–1.95	0.4–1.6	0.25–1.05	0.2–1.05	2.25 6.20

* Data from ref. 1; 37 samples taken over a 16-year period.

If the lower HPLC flavone values were due to incomplete extractions, recovery values would have to range between 25–80% to account for the differences observed. Since the data in Table IV indicate that recoveries are almost complete (with the exception of heptamethoxyflavone), other possibilities must be considered.

In Fig. 3, it can be seen that a UV-absorbing (and apparently non-fluorescing) impurity is found along with sinensetin in juice samples that have been treated with NaOH. This impurity is also reflected in sinensetin UV concentration levels (Table II) that are higher than either the corresponding fluorescence or non-treated juice sinensetin values. These falsely high UV concentration levels are in good agreement with those of Veldhuis *et al.*[1]. Thus, it is possible that the TLC–UV spectrophotometric procedure also incorporated this same impurity.

Tangeretin has the greatest discrepancy between concentrations determined by HPLC and TLC. TLC concentration levels are almost four times higher than those obtained by HPLC. A possible explanation can be seen from Fig. 3. It should be noted that two strong UV-absorbing (and non-fluorescing) peaks are formed as a result of the NaOH treatment and elute after tangeretin (at 20.85 and 22.51 min, respectively). Since the TLC procedure was done in the normal phase mode, these peaks would elute close to the solvent front and before tangeretin. (Long-wavelength UV irradiation was used to locate the weakly fluorescing tangeretin band on the streaked TLC plates.) Therefore, it is possible that some of this non-fluorescing material might have been scraped off with tangeretin. Thus, when the scrapings were redissolved, these compounds would cause an abnormally high UV absorbance which would then be calculated as tangeretin.

There is, however, good qualitative agreement between the two methods. In both methods nobiletin and sinensetin are the major methoxylated flavones found in orange juice accounting for approximately 60% of the total PMF content. Both methods find tangeretin to be the flavone in lowest concentration.

CONCLUSION

The HPLC procedure for the analysis of PMFs in OJ offers several advantages over the existing TLC–spectrophotometric method. Overall analysis time is less than half that required for the TLC method. Sample preparation time has been reduced by elimination of the NaOH treatment (which has been shown to be unnecessary). The actual analysis of the juice extract is much simpler and thus less subject to inaccuracies due to successive sample manipulations. Precision is excellent for a natural product analysis at the ppm level.

The use of combined UV and fluorescence detectors was invaluable in determining the presence of impurities and served as a cross check on quantitation. Using UV and fluorescence stop flow spectral data along with retention times, it was possible to identify unequivocally the five major methoxylated flavones from other peaks with similar retention times.

ACKNOWLEDGEMENT

The authors wish to acknowledge gratefully the invaluable technical assistance of Eleanor Case, Faye Martin and Gail Westberry. They also wish to acknowledge Martha Young for her help in preparing the manuscript.

The use of a Series 3 gradient pump from the Perkin-Elmer Corp. is gratefully acknowledged.

REFERENCES

1 M. K. Veldhuis, L. J. Swift and W. C. Scott, *J. Agr. Food Chem.*, 18 (1970) 590.
2 S. V. Ting, R. L. Rouseff, M. H. Dougherty and J. A. Attaway, *J. Food Sci.*, 44 (1979) 69.
3 L. J. Swift, *J. Agr. Food Chem.*, 13 (1965) 431.
4 L. J. Swift, *J. Agr. Food Chem.*, 15 (1967) 99.
5 S. M. Kupchan, J. R. Knox and M. S. Udayamurthy, *J. Pharm. Sci.*, 54 (1965) 929.
6 R. C. Robbins, *Int. J. Vitam. Nutr. Res.*, 47 (1977) 373.
7 L. R. Snyder, *J. Chromatogr. Sci.*, 7 (1969) 352.
8 T. C. Miller and L. R. Faulkner, *Anal. Chem.*, 48 (1976) 2083.
9 E. J. Bowen and A. H. Williams, *Trans Faraday Soc.*, 35 (1939) 65.
10 S. R. Bakalyar, M. P. T. Bradley and R. Honganen, *J. Chromatogr.*, 158 (1978) 277.
11 J. H. Tatum and R. E. Berry, *Phytochemistry*, 11 (1972) 2283.
12 T. R. Seshadri, *Tetrahedron*, 6 (1959) 169.

Journal of Chromatography, 176 (1979) 89–101
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,881

SYNTHESIS OF HIGHLY DIVERSIFIED CARRIER AMPHOLYTES

EVALUATION OF THE RESOLVING POWER OF ISOELECTRIC FOCUSING IN THE Pi SYSTEM (ALPHA-1-ANTITRYPSIN GENETIC POLYMORPHISM)

R. CHARLIONET, J. P. MARTIN, R. SESBOÜÉ, P. J. MADEC and F. LEFEBVRE
INSERM (U-78) sur la Génétique des Protéines Humaines, 76230 Bois-Guillaume (France)
(First received September 25th, 1978; revised manuscript received March 19th, 1979)

SUMMARY

The use of condensing reagents such as epoxypropanol, diepoxyoctane, acrylamide and N,N'-methylenebisacrylamide in the synthesis of carrier ampholytes increased the diversity of amphoteric components. The quality of these synthetic carrier ampholytes has been tested in the separation fo variants of alpha-1-antitrypsin, a genetic polymorphism called the Pi system. A resolving power of the order of 0.005 pH unit was obtained.

INTRODUCTION

Isoelectric focusing, a separation method for amphoteric molecules, is being employed more and more by investigators in a wide variety of fields. One of the more successful uses is the demonstration of genetic polymorphism of certain proteins. At present our main concern in this aspect is the Pi system (the different allotypes of human alpha-1-antitrypsin). The resolving power of isoelectric focusing, which is of prime importance for revealing slight electric differences between similar allelic products, depends mainly on the quality of the carrier ampholytes[1,2]. Two proteins can be completely separated only in the presence of other ampholytes with isoelectric points intermediate between the proteins and with good buffering capacity. These carrier ampholytes must fulfil certain criteria. They must have (1) adequate buffering capacity in the isoelectric state to determine the pH gradient even in the presence of proteins; (2) good conductivity at their p*I*'s; (3) sufficient solubility in water.

Vesterberg[3] synthesized a number of homologues and isomers of polyamine–polycarboxylic acid; these carrier ampholytes are now available commercially under their trade name Ampholine (LKB, Bromma, Sweden). Other carrier ampholytes are now also commercially available (Servalyt, Pharmalyte, Biolyt). Synthetic procedures were described in detail by Vinogradov *et al.*[4], Righetti *et al.*[5] and Grubhofer and Borja[6]. These reports represent real progress in the development of isoelectric focusing, for

they enable the synthesis of carrier ampholytes in one's own laboratory, the selection of the pH zone required for the study, and possibly improvement in their quality.

The now classical synthetic procedure involves treating aliphatic polyamine with acrylic acid. The primary and secondary amino groups can be added to the double bonds of the unsaturated acid so that a very large number of different polyaminoacids are obtained. We have introduced an additional variation by the use of condensing reagents such as epoxypropanol, diepoxyoctane, acrylamide and N,N'-methylenebis-acrylamide, in the attempt to form, after the addition on acrylic acid, a highly heterogeneous mixture of amphoteric components with a good repartition of the dissociation constants (pK) and with different isoelectric points.

Our aims were (1) to synthesize large amounts of carrier ampholytes, which would permit us to achieve better separation of the allotypes of alpha-1-antitrypsin polymorphism (Pi system) than would the commercially available ones and (2) to undertake genetic studies on a large scale. At least 24 alleles of the Pi system have been described, each of them characterized by a pattern of two major bands of focusing between pH 4.2 and 4.9. The isoelectric points of some of these alleles are very close to each other (*e.g.* PiM1, PiM3, and PiM2). Their identification is therefore very difficult if adequate carrier ampholytes are not used. Furthermore, new alleles may be disclosed if the resolving power of the method is improved.

We shall give in this report the whole synthetic procedure for the carrier ampholytes. Their quality will be described with reference to protein separation in the Pi system.

EXPERIMENTAL

Materials

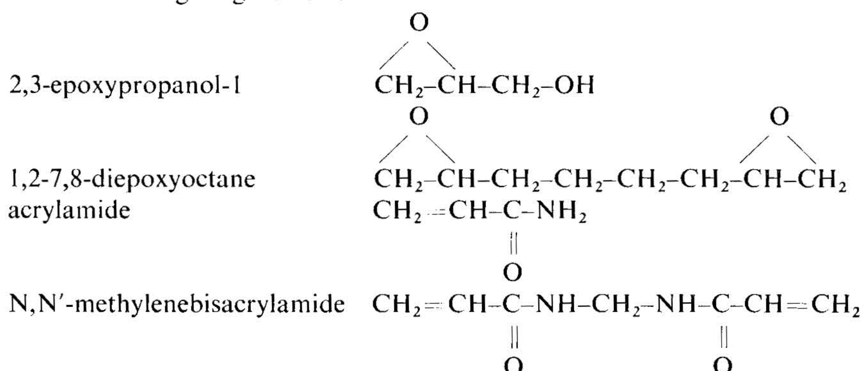
The chemical reagents and their sources were: triethylenetetramine (TETA) and tetraethylenepentamine (TEPA) from Aldrich (Beerse, Belgium); a mixture of hexamines called "N6" from BASF (Levallois Perret, France); 2,3-epoxypropanol-1, 1,2-7,8-diepoxyoctane, acrylic acid, riboflavin and sucrose from Merck (Darmstadt, G.F.R.); acrylamide and N,N'-methylenebisacrylamide (Bis) from OSI (Paris, France); trichloroacetic acid from Prolabo (Paris, France); Coomassie brilliant blue from Serlabo (Paris, France). Human sera were obtained from volunteers (Blood Transfusion Centre, Bois-Guillaume, France).

The polyamines used have amino groups two methylene groups apart for TEPA and TETA, and two or three methylene groups apart for the mixture of BASF hexamines. The pentaethylenehexamine which is often described as the basic amine for the synthesis of ampholytes was not employed in this study as we were not able to purchase it.

The polyamines must be heated (70°) under vacuum (20 mmHg) for 1 h just before use, because it is crucial to operate with carbonate-free polyamines. Bisacrylamide is recrystallized from acetone, and acrylamide from chloroform, as described by Loening[7]. Acrylic acid has to be distilled just before use under nitrogen and under reduced pressure in order to remove the polymerization inhibitor.

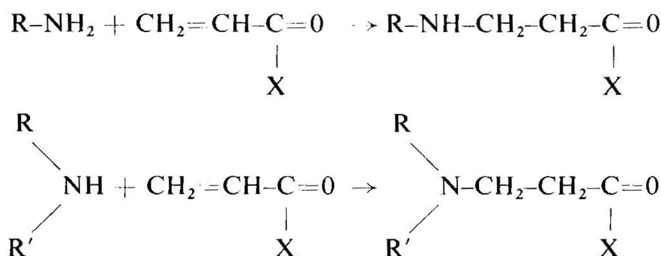
Methods

The condensing reagents are:



They were chosen for the facility of the reaction with amino groups and their hydrophylic properties.

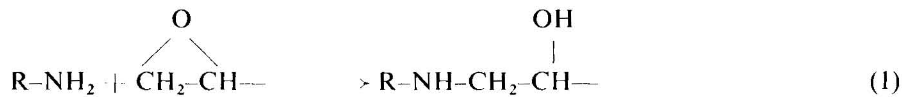
Reaction of acrylamide, bisacrylamide and acrylic acid. The nucleophilic groups of the polyamines can be added at the β-carbon atom of the α,β-unsaturated acid or amide[8]:

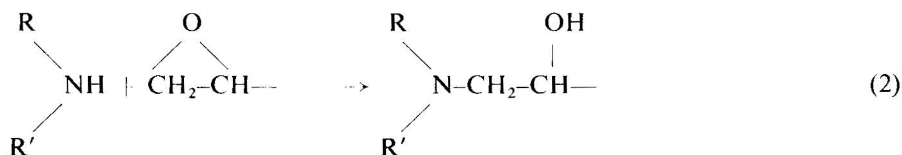


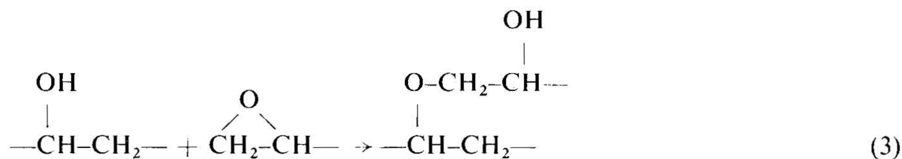
(X is OH or NHR)

We followed the reaction procedure described in detail by Righetti *et al*[5]. It is carried out in a four-necked flask equipped with a capillary for nitrogen flushing, a condenser fitted with a gas outlet and a stirrer. The polyamimes are diluted with the same volume of deionized water. Acrylic acid or a 30% acrylamide aqueous solution is added dripwise and Bis is added spoonwise. After the addition of reagents (1 h), the reaction is continued at 70° for 20 h.

Reaction of epoxypropanol and diepoxyoctane. The reaction of an epoxide group with a polyamine is expected to follow the reaction sequence[9]:







(3)

The reaction with a primary or secondary amine leads to the formation of a secondary hydroxyl group and respectively a secondary or tertiary amine (reactions 1 and 2). Another possible reaction is between the hydroxyl and an epoxide leading to crosslinking via formation of a new secondary hydroxyl group (reaction 3). However, Shechter *et al.*[10] concluded that the alcohol–epoxide reaction does not occur to a detectable extent.

The addition of polyamines to epoxide is carried out in the same flask as described above, under nitrogen and with continuous stirring. Epoxypropanol and diepoxyoctane are added dropwise and very slowly to the aqueous solution of polyamines. The reaction is continued at 50° for 20 h.

Preparation of ampholytes. The detailed method used to prepare ampholytes from polyamines, acrylic acid and condensing reagents, can be illustrated by the following three examples. In each one, the nitrogen/carboxyl ratio is *ca.* 7:5, in order to enhance the synthesis of ampholytes at pH 5.

Ampholytes I: (TEPA, TETA, N6) + (acrylic acid). Acrylic acid (44 g, 0.61 *M*) is added dropwise for 1 h, under an inert atmosphere, to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*) and deionized water (40 ml). The reaction mixture is heated under continuous stirring at 70° for 20 h. After cooling at room temperature, enough deionized water is added to make a 40% (w/v) solution of ampholytes. They are stored in sterilized inactinic* bottles at 4°.

Ampholytes II: (TEPA, TETA, N6) + (acrylamide) + (Bis) + (acrylic acid). A 30% solution in deionized water of acrylamide (50 ml, 0.21 *M*) is added dropwise for 1 h and under inert atmosphere to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*). The reaction continues under stirring at 70° for 4 h. The mixture is then cooled and Bis (6.5 g, 0.042 *M*) is added spoonwise; the reaction mixture is heated to 70° for 16 h. Acrylic acid is then added and the ampholytes stored under the same conditions as above.

Ampholytes III: (TEPA, TETA, N6) + (epoxypropanol) + (diepoxyoctane) + (acrylic acid). Epoxypropanol (15 g, 0.21 *M*) is added dropwise, very slowly, under an inert atmosphere, to a mixture of TEPA (20 g, 0.105 *M*), TETA (7.5 g, 0.05 *M*), N6 (10 g, 0.04 *M*) and deionized water (40 ml). The reaction continues under stirring at 50° for 4 h. The mixture is then cooled and diepoxyoctane (6 g, 0.04 *M*) added dropwise. The reaction mixture is heated to 50° for 16 h. Acrylic acid is then added and ampholytes stored under the same conditions as above.

Fractionation of carrier ampholytes. Only ampholytes focusing between pH 4.2 and 5.4 are useful for the study of the Pi system. They are isolated by a preparative isoelectric focusing technique in open horizontal layers of granulated gel, as described by Radola[11].

The granulated gel used as the stabilizing medium is Sephadex G-15 (Pharmacia, Uppsala, Sweden). This is preferred to Sephadex G-75 (or G-100) superfine

* inactinic — not permitting the chemical action of radiant energy.

used in preparative protein because of its remarkable mechanical properties. We used the electrofocusing kit for granulated gel, the Multiphor and the model 2103 power supply from LKB.

The fractionation procedure is as follows. A slurry of Sephadex G-15 (100 ml) is filtered on a glass filter funnel and washed with deionized water. 25 ml of a 40% solution of synthetic ampholytes is mixed with the filtered gel in a beaker. The homogenized suspension is then poured on to a horizontal tray with three layers of wet strips at each end. The suspension spreads evenly. The excess of water is evaporated with a light stream of air. At the end of this process (which is easy to determine with Sephadex G-15), the cathode and anode strips, respectively soaked with sodium hydroxide (1 *M*) and phosphoric acid (1 *M*), are positioned and the tray is put on the Multiphor unit. The limiting electrical conditions are 11 W, 20 mA, 1500 V. A run of 48 h is needed for a good separation, after which a grid allows the focused ampholytes to be collected. Each separated zone is put in a vial and resuspended with deionized water. The pH is then measured with a combined glass microelectrode fitted on a digital pH meter (Metrohm Herisau). The ampholytes can be eluted very easily by filtration. They are concentrated on a rotative evaporator and kept as 40% solution (w/v) in sterilized inactinic bottles at 4°.

In this study, we used carrier ampholytes encompassing three pH ranges: 4.2–5.3, 4.3–4.9 and 4.4–4.7. The larger ranges were separated directly from the solution of synthetic ampholytes. The narrower one required further fractionation from ampholytes of pH range 4.2–5.3. The pH range 4.3–4.9 was also obtained by fractionating commercial ampholytes (Ampholine LKB, 3.5–5.0).

Analytical isoelectric focusing on polyacrylamide gel plate. This is performed on a LKB Multiphor apparatus. The gel is made to a final concentration of acrylamide 5%, carrier ampholytes 1% (w/v) and sucrose 12% (w/v). Polymerisation is accomplished with riboflavin 0.04% (v/v) and UV light for 1 h. A prerun is carried out for 1 h and, after application of the samples, isoelectric focusing is performed for 2 h with the following maximal electrical conditions: 10 W, 10 mA and 1600 V. The pH gradients in the gel slabs are measured with a contact electrode (Ingold, Sofranie, France) and read on a digital pH meter. Fixation with trichloroacetic acid, staining with Coomassie blue and destaining are carried out as described by Vesterberg *et al.*[12].

Conductivity measurements. These were carried out, as described by Righetti *et al.*[13], to compare the conductivity as a function on the pH gradients between synthetic and commercial carrier ampholytes. After focusing, the gels were cut carefully into 6 × 0.5 × 0.1 cm slices, and each fraction was eluted for 4 h in 4 ml of distilled water. Conductivity measurements were made with a Metrohm Herisau conductimeter E382 (Sofranie, France). The pH measurements of each fraction were made with a combination electrode (Ingold).

RESULTS

Fig. 1 shows the pattern of several alpha-1-antitrypsin phenotypes (BM3, IM2, M1S, M1Z, Z, S, M1M3, M1M2, M2M3) obtained with ampholytes I (Fig. 1a) and ampholytes III (Fig. 1b). Both ampholytes have been fractionated to give pH gradients in the range 4.2–5.3.

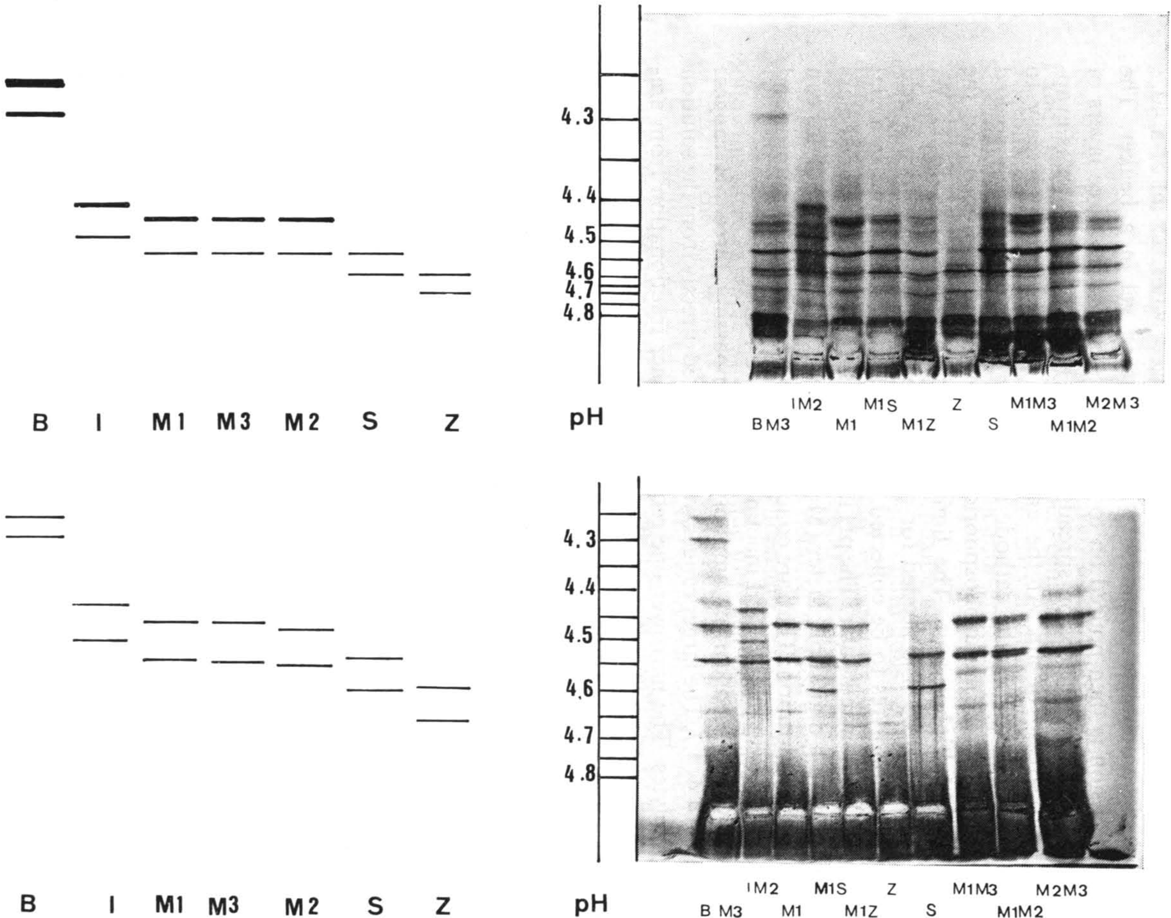


Fig. 1. Results obtained with (a) ampholytes I and (b) ampholytes III, following fractionation to give pH gradients in the range 4.2–5.3. On the right are the isoelectrofocusing patterns of several alpha-1-antitrypsin phenotypes. In the middle is the pH scale as measured by a contact electrode on the polyacrylamide gel plate after focusing. On the left are the graphs of the two major bands (namely the 4 and 6 bands) of the alpha-1-antitrypsin patterns for several alleles.

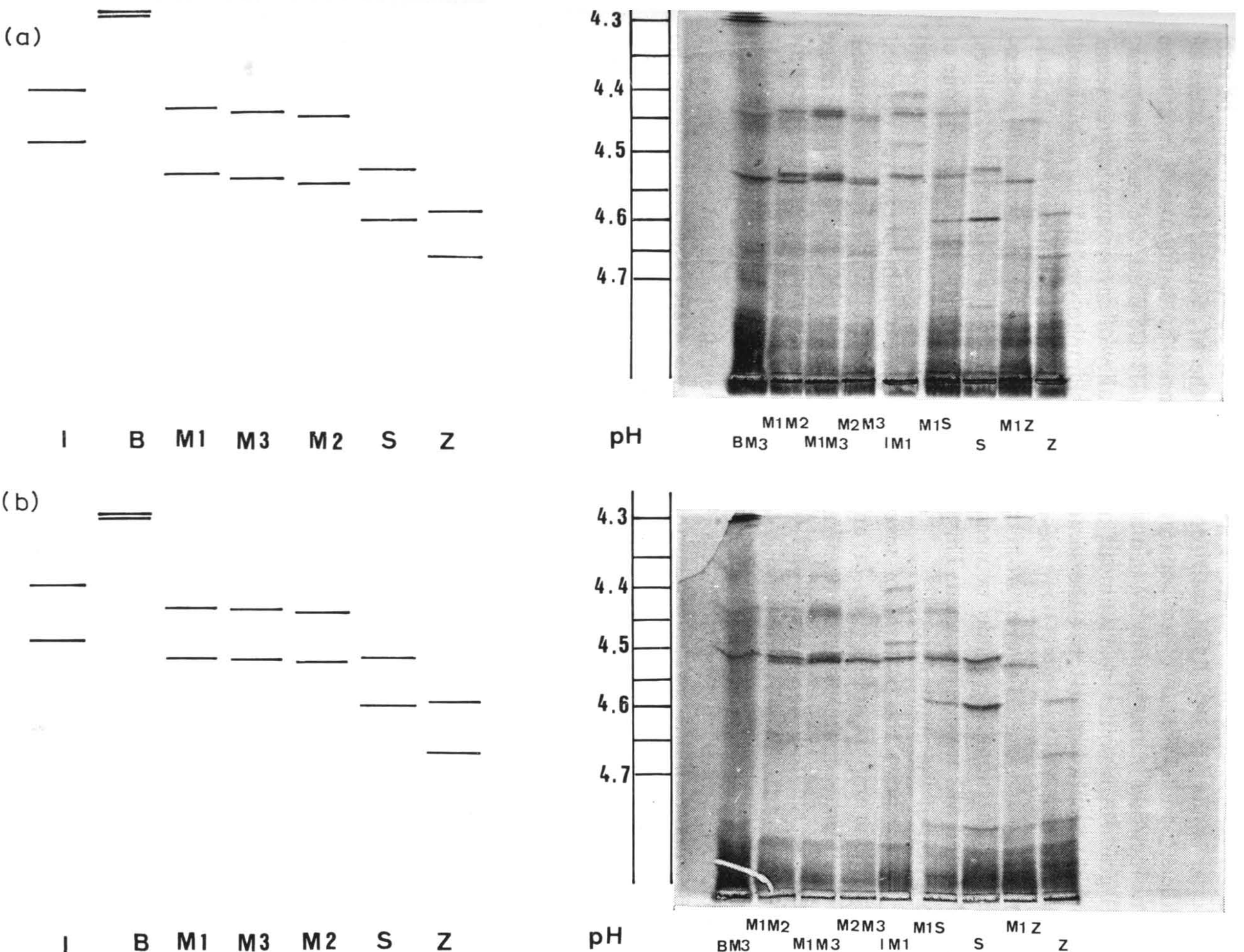


Fig. 2. Results obtained with (a) ampholytes III and (b) commercial ampholyte (Ampholine LKB), following fractionation to give pH gradients in the range 4.3–4.9. The details are the same as for Fig. 1.

It is evident that ampholytes I are quite inadequate for determining alpha-1-antitrypsin phenotypes. There are gaps in distribution within the pH range 4.2–5.3, the pH gradient is not linear, and the quality seems to be very poor. It can be seen that some bands are not at all well focused, *e.g.* the M_4 bands. In the pattern of each allele, bands '4' and '6' are separated but cannot be distinguished from other proteins focusing in the vicinity. When ampholytes III are used, the gaps disappear, the pH gradient becomes nearly linear, and the quality of carrier ampholytes seems improved. (Ampholytes III give the same results as ampholytes II.)

Synthetic ampholytes III can be used for the determination of the alpha-1-antitrypsin phenotypes. However, from this zone of ampholyte fractionation, a clear-cut distinction cannot be made between the M1 and M3 or M3 and M2 alleles. Only M1 and M2 are distinguished. This explains the necessity of a narrower zone of fractionation of the ampholytes.

Fig. 2 shows the patterns of several alpha-1-antitrypsin phenotyes obtained from synthetic ampholytes III (Fig. 2a) and from commercial ampholytes (Ampholine LKB) (Fig. 2b), each following further fractionation to give pH gradients in the range 4.3–4.9. The separations of the 4 and 6 bands of M1, M3, and M2 are excellent for ampholytes III. In the same manner, S_4 and M_6 bands, Z_4 and S_6 bands are very clearly distinguished. The resolving power reached by ampholytes III in this case can be estimated at 0.005 pH units or less. The commercial product used in the same pH range does not give as good separtion as ampholytes III (Fig. 2b).

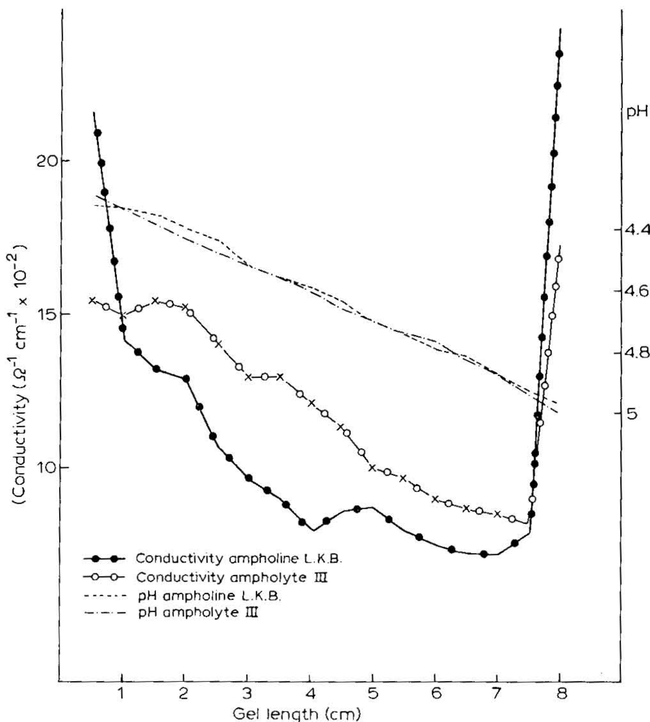


Fig. 3. Comparison of the conductivity and the pH gradient profile for ampholytes III and Ampholine LKB, fractionated in the pH range 4.3–4.9.

Some physicochemical data relating to ampholytes III and Ampholine (LKB), both of them fractionated in the pH range 4.3–4.9, are compared in Fig. 3 (conductivity and pH gradient profiles). Ampholytes III have a slightly better conductivity throughout the pH gradient considered than Ampholine LKB. When ampholytes III are subjected to a second step of purification to obtain the very narrow pH range 4.4–4.7, the separations of M1, M2 and M3 phenotypes are impressive (Fig. 4). Furthermore, some new alleles are revealed. Although the identification of certain of these PiM heterozygotes is unreliable, the corresponding new alleles are distinguishable when focused side by side. The differences between their p*I*'s can be estimated to be less than 0.001 pH unit. The family studies of these new variants will be published elsewhere.

DISCUSSION

The resolving power

Synthetic ampholytes III used in optimum conditions can separate proteins whose p*I*'s are less than 0.005 pH unit apart (*e.g.*, $M1_6$ and $M3_6$ or $M2_6$ and $M3_6$) and allows us to distinguish proteins differing only by 0.001 pH unit. Such a resolving power has not been realized previously for the Pi system.

Allen *et al.*[14] determined alpha-1-antitrypsin phenotypes with isoelectric focusing on polyacrylamide gel slabs containing commercial carrier ampholyte (Ampholine LKB 3.5–5.0). They were able to separate the basic variants and to measure their isoelectric points. Since then, this method has been widely used. Nevertheless, the resolving power of this technique was without doubt overstated when Allen claimed that proteins with p*I*'s differing by 0.0025 pH unit could be resolved. In fact, M1 and M2, which differ by only 0.01 pH unit, were not distinguished.

The separation of M1 and M2 was initially reported by Frants and Eriksson[15], and M3 was revealed a few months later by one of us[16] and confirmed recently by Frants *et al.*[17]. These improvements in resolving power were realized with Ampholine LKB by using several methods of decreasing the slope of the pH gradients. Two of them have been used in our laboratory: the mixture of Ampholine LKB 4–6 and 3.5–5.0, and the fractionation of Ampholine LKB 3.5–5.0. This study shows that our synthetic carrier ampholytes III compare favorably with Ampholine LKB in physicochemical properties and in the resolving power.

Frants *et al.*[17] have chosen another interesting way to influence the slope of the pH gradient: the addition of amphoteric substances called 'separators' by Caspers and co-workers[18] to separate M1, M2 and M3. These proteins have also been recently claimed by Kueppers and Christopherson[19] to be separated with Ampholine LKB 3.5–5.0. However, it seems to us that they misinterpreted the phenotypes of the serum used. On the one hand, the distance between the so-called $M2_6$ and $M1_6$ bands is far too big in comparison with the distance between $M1_6$ and $M1_4$ bands. On the other hand, the excess of carrier ampholyte they used would broaden the focused bands rather than improve the resolving power.

Increasing the diversity of carrier ampholyte species

The gaps in the distribution of ampholytes I, which are seen in the pH range 4.2–5.3, are filled when ampholytes II and III are used. The introduction of epoxy-

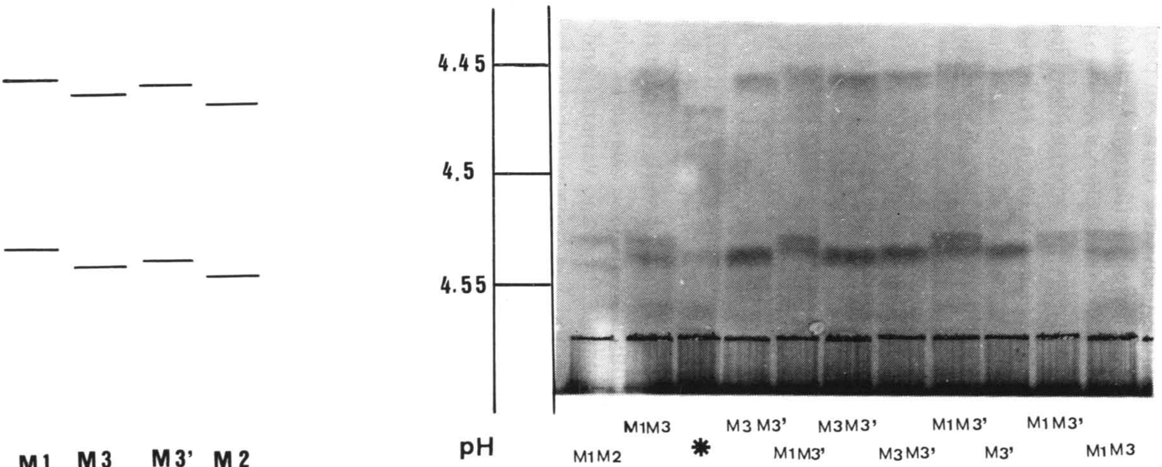
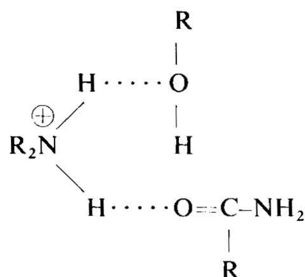


Fig. 4. Results obtained with ampholyte III subjected to a second step of purification to obtain the pH range 4.4–4.7. The details are the same as for Fig. 1. One of the new variants discovered can be seen; we have provisionally called it M3′. Although the identification of the Pi M3M3′ heterozygotes is unreliable, the corresponding alleles are distinguishable when focused side by side. The asterisk refers to an old serum previously phenotyped Pi M3′.

propanol (or acrylamide) and diepoxyoctane (or bisacrylamide) is sufficient for the synthesis of new species of ampholytes with evenly distributed isoelectric points and with a good repartition of the dissociation constants. Two explanations may be put forward to justify this increase of diversity of ampholyte species.

The main polyamine used in this study is TEPA, which is a pentamine with seven nucleophiles (>N-H). The number of acrylic acid molecules to be coupled to one molecule of TEPA for the synthesis of ampholytes having isoelectric points in the vicinity of pH 5 might be estimated at 4. From a strictly formal point of view, there are theoretically ten different ampholyte species and 24 different substrates for further addition reactions, which can be synthesized using these conditions. Neutral reagents such as epoxide or acrylamide are added. They will not notably modify the isoelectric point of the ampholytes, so that we can continue to consider the ratio carboxylic/TEPA to be 4. With the quantity of epoxypropanol (or acrylamide) added (epoxide/TEPA = 1), it might be expected that mainly three ampholyte populations would be synthesized: ampholytes having reacted with zero, one or two molecules of epoxypropanol. Again from a strictly formal point of view, we could theoretically get a total of 58 different ampholyte species, and 89 different substrates for further addition reations. If we now consider the addition of a coupling reagent such as diepoxyoctane (or bisacrylamide), in the ratio diepoxyoctane/TEPA = 1/5, the most probable configuration we can obtain for the molecules that have reacted is the crosslinking of two TEPA by one dialcohol–octane bridge. This would lead to 3916 different ampholyte species. This number has been calculated from C_2^{89}, which represents the possible set of two things selected from 89, irrespective of arrangement within the set. In fact, we use a mixture of several polyamines in the synthesis of ampholytes; thus other combinations could be envisaged, so that the number of different ampholyte species with isoelectric point near pH 5 should be much greater than 3916. However, this number should be considerably reduced by the fact that, in TEPA for instance, the seven nucleophiles do not possess the same reactivity. The probability that some of them may react will be very low, so that the corresponding ampholyte species should be practically non-existent. It should also be kept in mind that most of the different synthesized ampholyte species, although not quite identical in configuration, should have nearly indistinct isoelectric points.

The increase of diversity of ampholyte species might also be explained by the possibility that the introduction of the alcohol or amide groups changes the strength of the nitrogenous bases. This could be due to the fat that the basic strength of an amine is determined, among other factors, by the extent to which the cation, formed by uptake of a proton, can become stabilised. In a molecule in which hydroxyl (or amide) groups are present together with amino groups, such a stabilisation is possible possible via hydrogen bonding in the following way[20]:



The effect of this intramolecular hydrogen bonding on the constants of dissociation of the basic groups and on the isoelectric points of the final carrier ampholytes is difficult to quantify. Furthermore, hydroxyl (or amide) should compete with carboxylic groups in the formation of hydrogen bonds. Nevertheless, the fact that both kinds of reagents, epoxypropanol and diepoxyoctane (or acrylamide and bisacrylamide), are necessary to fill the gaps indicates that the two proposed explanations are complementary.

It should also be noted that the addition of epoxypropanol and diepoxyoctane has to be carried out before the addition of acrylic acid, otherwise the quality of ampholytes is not improved enough. This is somewhat surprising since we could have expected another type of reaction between the epoxide and the carboxylic groups, leading with tertiary amine as catalyst to an hydroxyl ester.

CONCLUSION

We have tried to improve the quality of carrier ampholytes. The introduction of coupling reagents such as epoxypropanol, diepoxyoctane, acrylamide and bisacrylamide during their synthesis has proved to be useful. However, the polyamines employed are not the best for synthesizing ampholytes. It has been shown that TEPA and TETA on their own give very poor mixtures of ampholytes[3,5]. The ampholytes obtained with the mixture of hexamines 'N6' provided by BASF do not appear to be better than TEPA ampholytes, and we have confirmed that our ampholytes I are not suitable for the determination of the Pi system by isoelectric focusing. Curing of pentaethylenehexamine (which has proved to be very good for ampholyte synthesis) with the coupling reagents used in this study should give excellent results. Unfortunately, it is now practically unavailable.

Other amines could be tried for this purpose. Condensation of ethyleneimine with propylenediamine[6] could give a very good starting material. Other approaches to the improvement of the properties of carrier ampholytes could be achieved by looking for different acids (itaconic acid[13], propanesulphone and chloromethylphosphoric acid[6]) or other coupling reagents.

It is worth noting that in this study the synthesis of ampholytes has been conducted in such a way as to favour ampholytes with isoelectric points in the vicinity of pH 5. The synthesis of carrier ampholytes of different isoelectric points should be carried out in different conditions: the nature and the proportion of the reagents must be adapted empirically. We wish to add that this necessary adaptation can easily be made in a standard biochemical laboratory.

We succeeded in synthesizing large amounts of carrier ampholytes, which allowed us to study the Pi system more thoroughly than did the commercially available ones. The improvement of resolving power is of prime importance to reveal new alleles. We are convinced that the limit of isoelectric focusing as the method for protein separation has not yet been reached. Further research on the chemical nature of ampholytes must be undertaken. Synthetic carrier ampholytes fractionated in narrow pH range by means of preparative electrofocusing might be considered as quite versatile 'separators'.

ACKNOWLEDGEMENTS

This work was supported in part by INSERM, Grant No ATP 55-77-87 and the University of Rouen; one of us, P. J. Madec, is a scientist of Institut National Supérieur de Chimie Industrielle de Rouen, Département of Pr. Maréchal.

REFERENCES

1 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325.
2 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456.
3 O. Vesterberg, *Acta Chem. Scand.*, 23 (1969) 2653.
4 S. N. Vinogradov, S. Lowenkron, M. R. Andonian, J. Bagshaw, K. Felgenhauer and S. J. Pak, *Biochem. Biophys. Res. Commun.*, 54 (1973) 501.
5 P. G. Righetti, M. Pagani and E. Gianazza, *J. Chromatogr.*, 109 (1975) 341.
6 N. Grubhofer and C. Borja, in B. J. Radola and D. Graesslin (Editors), *Proc. Int. Symp. on Electrofocusing and Isotachophoresis, Hamburg, August 2–4, 1976*, Walter de Gruyter, Berlin, New York, 1977, Part I, p. 111.
7 U. E. Loening, *Biochem. J.*, 102 (1967) 251.
8 P. Sykes, *A guidebook to mechanism in organic chemistry*, Longmans, London, 2nd ed., 1965, p. 155.
9 W. G. Potter, *Epoxide Resins*, Butterworths, London, 1970, p. 40.
10 L. Shechter, J. Wynstra and P. P. Kurjy, *Ind. Eng. Chem.*, 48 (1956) 94.
11 B. J. Radola, in N. Catsimpoolas (Editor), *Isoelectric focusing*, Academic Press, New York, San Francisco, London, 1976, Ch. 6, p. 119.
12 O. Vesterberg, *Biochim. Biophys. Acta*, 257 (1972) 11.
13 P. G. Righetti, L. Balzarini, E. Gianazza and O. Brenna, *J. Chromatogr.*, 134 (1977) 279.
14 R. C. Allen, R. A. Harley and R. C. Talamo, *Amer. J. Clin. Path.*, 62 (1974) 732.
15 R. R. Frants and A. W. Eriksson, *Hum. Hered.*, 26 (1976) 435.
16 T. Genz, J. P. Martin and H. Cleve, *Hum. Genet.*, 38 (1977) 325.
17 R. R. Frants, G. T. Noordhoek and A. W. Eriksson, *Scand. J. Clin. Lab. Invest.*, 38 (1978) 457.
18 R. K. Brown, M. L. Caspers and S. N. Vinogradov, in B. J. Radola and D. Graesslin (Editors), *Proc. Int. Symp. on Electrofocusing and Isotachophores, Hamburg, August 2–4, 1976*, Walter de Gruyter, Berlin, New York, 1977, Part I, p. 87.
19 F. Kueppers and M. J. Christopherson, *Amer. J. Hum. Genet.*, 30 (1978) 359.
20 P. Sykes, *A guidebook to mechanism in organic chemistry*, Longmans, London, 2nd ed., 1965, p. 50.

Journal of Chromatography, 176 (1979) 103–108
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,894

DETERMINATION OF SPECTINOMYCIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

HARVEY N. MYERS and JAMES V. RINDLER
The Upjohn Company, Kalamazoo, Mich. 49001 (U.S.A.)
(Received March 27th, 1979)

SUMMARY

A high-performance liquid chromatographic method for the quantitative determination of spectinomycin in the presence of actinospectinoic acid and actinamine is described. A combination of paired ion chromatography, post column oxidation, post column derivatization and fluorometric detection is employed. Total assay time is less than 15 min. The assay was designed for determining spectinomycin in samples of spectinomycin finished products, process samples and fermentation beers. Quantitative results from this procedure are compared to those obtained by a turbidimetric microbiological assay and are in good agreement.

INTRODUCTION

Spectinomycin is a broad-spectrum aminocyclitol antibiotic isolated from fermentation broth of *Streptomyces spectabilis*[1–4]. This antibiotic is polyfunctional and is stable only within a narrow pH range. Spectinomycin and most of its degradation products are water-soluble compounds which lack UV-absorbing chromophores. Preparation of homogenous UV-absorbing derivatives of these substances in a quantitative manner has been reported to be a very arduous task[5]. As a result, a rapid and reliable, chromatographic method of analysis has not been heretofore developed.

Two chromatographic methods of analyses for determining the purity of modified spectinomycins and degradation products have been employed in this laboratory[5,6], These methods were high-performance liquid chromatography (HPLC) and gas chromatography (GC). In the former, the compounds were resolved on ion-exchange resin and detected by a differential refractometer or a photoelectric polarimeter. In the latter the compounds were derivatized with hexamethyldisilazane, chromatographed on an SE-30 stationary phase and detected by a flame ionization detector. Since these procedures lacked speed, sensitivity and precision, an alternative chromatographic approach for the quantitative determination of spectinomycin and its degradation products was investigated.

Recently, HPLC methods using fluorometric detection for gentamicin[7,8] and kanamycin[9] have come to our attention and have led us to investigate the use of a simi-

lar procedure for spectinomycin. This paper describes the paired ion HPLC method with fluorometric detection for the determination of spectinomycin in the presence of actinospectinoic acid and actinamine.

EXPERIMENTAL

Equipment

A schematic diagram of the chromatographic apparatus is shown in Fig. 1. A Spectra-Physics Model 740B Pump was used to deliver the mobile phase. The assay was run on a Merck LiChrosorb RP-8 Hibar® II column (25 cm × 4.6 mm I.D.) with a precolumn (4.3 cm × 4.2 mm I.D.) packed with Merck Perisorb® RP-8 packing. Samples were injected using a Valco CV-6UHPa-N60 injection valve with a 15-μl injection loop. Two Milton-Roy Model 196-89 Minipumps equipped with Glenco pulse dampeners were used to deliver the oxidizing reagent and the fluorogenic reagent. Precolumns packed with Applied Sciences glass beads (170–230 mesh) were installed in the reagent lines to generate back pressure for the pulse dampeners. The oxidant was mixed with the column effluent in a mixing tee (Cheminert CJ 3031 fitting). This stream then passed through a thermostated reaction coil (2.0 m × 0.5 mm I.D. PTFE tubing) at 100°. The oxidized column effluent was mixed with the

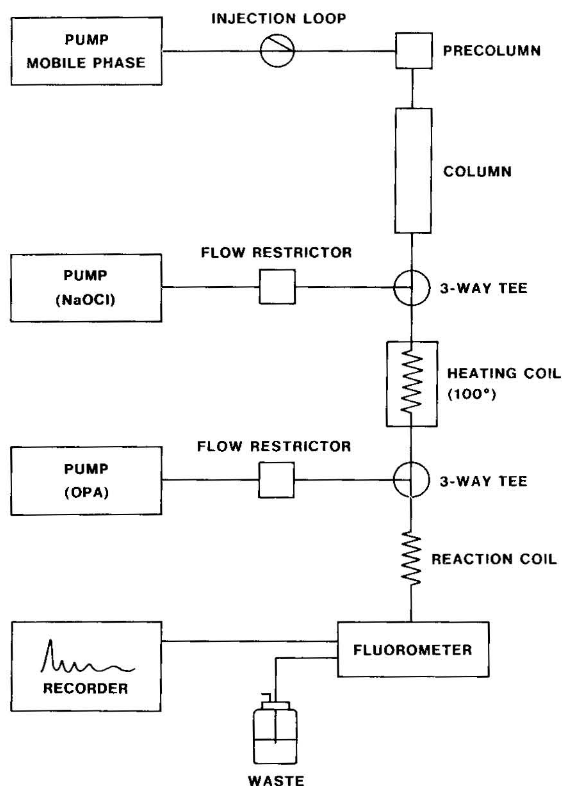


Fig. 1. Schematic diagram of the HPLC apparatus used for spectinomycin determinations.

fluorogenic reagent in a second mixing tee (Cheminert fitting CJ 3031) and then was passed through a second reaction coil (2.0 m × 0.5 mm I.D. PTFE tubing) at ambient temperature. The effluent then was passed into the Perkin-Elmer 204A fluorometer. Excitation wavelength was 350 nm and emission wavelength was 450 nm. The detector signal was processed using the Hewlett-Packard 3354A data system and a Hewlett-Packard 7153A recorder.

Reagents

Spectinomycin hydrochloride*, actinamine and actinospectinoic acid were obtained from The Upjohn Company (Kalamazoo, Mich., U.S.A.), *o*-phthalaldehyde (Fluoropa, manufactured by Durrum) from Pierce (Rockford, Ill., U.S.A.), 2-mercaptoethanol from Sigma (St. Louis, Mo., U.S.A.), sodium heptanesulfonate from Regis (Morton Grove, Ill., U.S.A.), and sodium hypochlorite (Clorox®, manufactured by The Clorox Company, Oakland, Calif., U.S.A.) from a local vendor. Water was double distilled. All other chemicals were of reagent grade. *o*-Phthalaldehyde reagent solution (OPA) was prepared by the method of Benson and Hare[10]. The oxidizing reagent contained 0.01 *M* sodium hypochlorite in 0.4 *M* potassium borate buffer, pH 10.4. Solutions of antibiotic and by-products were freshly prepared in distilled water at concentrations of 50–900 μg/ml.

Mobile phase

The mobile phase contained 0.02 *M* sodium heptanesulfonate, 0.2 *M* sodium sulfate and 0.1 % acetic acid in water. The mobile phase, after it has been filtered and degassed, was pumped through the column at a flow-rate of 2 ml/min. The oxidizing reagent and the fluorogenic reagent each are pumped into the mixing tees at a flow-rate of 0.5 ml/min.

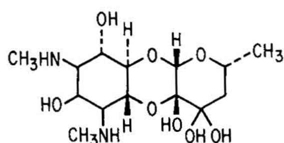
RESULTS AND DISCUSSION

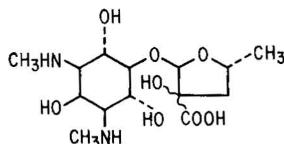
Unlike other aminoglycoside antibiotics for which fluorometric detection has been used[7–9], spectinomycin contains no primary amino groups[10]. There are two secondary amino groups in the actinamine portion of this molecule (see Fig. 2)[11]. Therefore, this antibiotic does not react directly with *o*-phthalaldehyde to form a fluorescent species.

In order to detect spectinomycin via fluorescence, we considered two alternatives. The first possibility was to find a fluorogenic reagent that would react with secondary amines in aqueous solution. Dansyl chloride is the most commonly used fluorogenic reagent for reaction with secondary amines; however, it and its by-products are fluorescent. The second possibility was to convert the secondary amine to a primary amine and then react it with *o*-phthalaldehyde. We chose to pursue the latter course.

Several reports in the literature described the transformation of secondary amino acids (*e.g.*, proline, hydroxyproline) to primary amines and their subsequent reaction with fluorogenic reagents[12–14]. These secondary amino acids, when treated with either chloramine-T, N-chlorosuccinimide, sodium hypochlorite or other sources

* The registered U.S. trademark of The Upjohn Company for spectinomycin, formerly known as actinospectacin, is Trobicin. Additional trademarks include Togamycin and Stanilo.





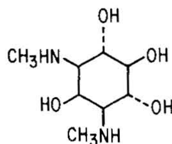


Fig. 2. Structures of spectinomycin and two of its degradation products.

of active halogen, underwent oxidative decarboxylation. The resulting imines were hydrolyzed to primary amines which were subsequently treated with fluorogenic reagents to form fluorescent compounds.

By analogy, spectinomycin was allowed to react with sodium hypochlorite. The degradative product was then treated with *o*-phthalaldehyde. The reaction mixture showed fluorescence with a response proportional to the spectinomycin content.

Once the problem of detecting the antibiotic was solved, the use of several different alkanesulfonate counter ions (C_5–C_7) in the mobile phase was investigated. It was concluded that sodium heptanesulfonate yielded the best separation of spectinomycin from its by-products. Typical chromatograms of a sample of spectinomycin and of spectinomycin fermentation beer are shown in Fig. 3 and 4.

It should be noted that there is a small peak in the chromatogram in Fig. 3 labeled "unknown". When a freshly prepared solution of the antibiotic is chromatographed immediately, the "unknown" peak is barely detectable. After 30 min this peak reaches a steady state which amounts to *ca.* 8% of the spectinomycin peak. This unknown component might be one of the spectinomycin diastereomers postulated by Foley and Weigele[15,16].

Calibration of assay

Several water-soluble secondary amino compounds were investigated for use as internal standards; however, no suitable substance was found. The assay was quantitated as an external standard method. Calibration curves were constructed for spectinomycin, actinospectinoic acid and actinamine. These curves were found to be linear in the region investigated from 0.05 mg/ml to 0.9 mg/ml. The precision of the assay expressed as relative standard deviation for repeated injections of standard samples was found to be 2.2% for spectinomycin, 1.7% for actinamine and 1.2% for actinospectinoic acid.

Ten crystalline samples of spectinomycin hydrochloride were analyzed using this HPLC procedure. These sample results were compared to those obtained using a

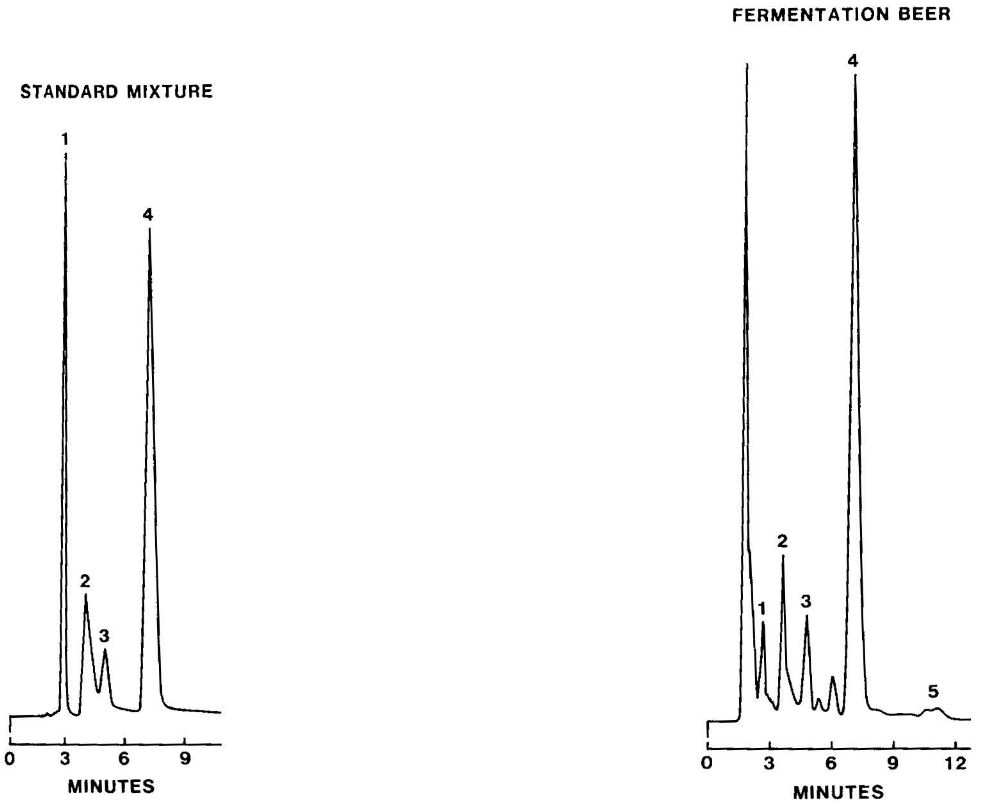


Fig. 3. Typical chromatogram of a standard mixture of spectinomycin (4), actinospectinoic acid (2), actinamine (1) and unknown (3).

Fig. 4. Typical chromatogram of spectinomycin fermentation beer. Peaks as in Fig. 3 except for dihydrospectinomycin (5).

TABLE I

COMPARISON OF THE DETERMINATION OF SPECTINOMYCIN BY HPLC AND MICROBIOASSAY

Sample	*Autoturbidimetric microbioassay (μg/mg)*	*HPLC (μg/mg)*
1	681	675
2	670	648
3	636	640
4	587	596
5	595	612
6	599	582
7	558	599
8	615	637
9	598	598
10	594	626
	Mean 613	621

turbidimetric microbiological assay. These data are summarized in Table I. The results from both assay methods agree well. The HPLC assay has advantages of speed, accuracy and sensitivity.

ACKNOWLEDGEMENT

We thank Dr. Fred Sancilio for a copy of the preprint entitled Gentamicin-C-Component Ratio Determination by High Pressure Liquid Chromatography. We also wish to express our gratitude to Drs. H. K. Jahnke, J. C. Knight and W. M. Knoll for helpful suggestions and for reviewing the manuscript.

REFERENCES

1 P. F. Wiley, A. D. Argoudelis and H. Hoeksema, *J. Amer. Chem. Soc.*, 85 (1963) 2652.
2 D. J. Mason, A. Dietz and R. M. Smith, *Antibiot. Chemother.*, 11 (1961) 118.
3 M. E. Bergy, T. E. Eble and R. R. Herr, *Antibiot. Chemother.*, 11 (1961) 661.
4 A. C. Sinclair and A. F. Winfield, *Antimicrob. Ag. Chemother.*, (1962) 503.
5 J. C. Knight, *J. Chromatogr.*, 136 (1977) 432.
6 L. W. Brown and P. B. Bowman, *J. Chromatogr. Sci.*, 12 (1974) 373.
7 J. P. Anhalt ,F. D. Sancilio and T. McCorkle, *J. Chromatogr.*, 153 (1978) 489.
8 J. P. Anhalt, *Antimicrob. Ag. Chemother.*, 11 (1977) 651.
9 D. L. Mays, R. J. Van Apeldoorn and R. G. Lauback, *J. Chromatogr.*, 120 (1976) 93.
10 J. R. Benson and P. E. Hare, *Proc. Nat. Acad. Sci., U.S.*, 72 (1975) 619.
11 T. G. Cochran, D. J. Abraham and L. L. Martin, *J. Chem. Soc., Chem. Commun.*, (1972) 494.
12 M. Roth, *Anal. Chem.*, 43 (1971) 880.
13 M. Roth and A. Hampai, *J. Chromatogr.*, 83 (1973) 353.
14 M. Weigele, S. DeBernardo and W. Leimgruber, *Biochem. Biophys. Res. Commun.*, 50 (1973) 352.
15 K. L. Rinehart, Jr., University of Illinois-Champaign Urbana, personal communication, 1978.
16 L. Foley and M. Weigele, *J. Org. Chem.*, 43 (1978) 4355.

Journal of Chromatography, 176 (1979) 109–117
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,895

USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR THE DETERMINATION OF PROVITAMIN A CAROTENES IN TOMATOES*

MONA ZAKARIA and KENNETH SIMPSON

Department of Food Science and Technology, Nutrition and Dietetics, University of Rhode Island, Kingston, R.I. 02881 (U.S.A.)

PHYLLIS R. BROWN

Department of Chemistry, University of Rhode Island, Kingston, R.I. 02881 (U.S.A.)

and

ANTÉ KRSTULOVIC

Manhattanville College, Purchase, N.Y. 10577 (U.S.A.)

(First received December 19th, 1978; revised manuscript received March 28th, 1979)

SUMMARY

The usual methods for provitamin A evaluation of foods convert the total pigment amount, determined spectrophotometrically, into vitamin A units. Since the totally inactive lycopene is the major carotenoid in the tomato, such readings result in erroneously high provitamin A values.

In view of the recent development of chemically bonded, reversed-phase, microparticulate packings and their use in high-performance liquid chromatography which combines highly accurate and reproducible resolution with the speed and ease of operation, a new method using such a system was developed to isolate carotenoid pigments from tomato samples. A 15-min column separation was thus achieved, dramatically decreasing the analysis time of the classical open column chromatographic procedures, which often result in unresolved and altered fractions due to long-term exposure to oxygen, light, solvents and sometimes adsorbent.

β-Carotene and lycopene were determined and quantitated in six tomato samples. β-Carotene, 100% vitamin A-active, was expressed in International Units of vitamin A. The newly developed method gives a more reliable evaluation of the fruit potency in vitamin A than the methods of the Association of Official Analytical Chemists currently used for food composition tables.

INTRODUCTION

Differences in the biopotency of carotenoids as vitamin A precursors result from their individual structures. The β-ring present in retinol is essential for their

* R.I. Agricultural Experiment Station Contribution No. 1839.

activity. β-Carotene, having two such rings, is considered 100% vitamin A-active. α-Carotene is only one half as potent while acylcic carotenes such as lycopene, also naturally present in foods, are totally inactive (Fig. 1). The determination of provitamin A levels in foods hence requires the isolation and accurate quantitation of those carotenes with biological significance. Few of the analytical methods available are suitable for this purpose: open-column chromatography, besides being time-consuming and allowing long-term exposure of carotenoids to oxygen, light, adsorbents and solvents, often fails in resolving the most potent vitamin A precursor, all-*trans*-β-carotene, from its less active geometrical isomer, α-carotene (1); both this method and thin-layer chromatography lack in reproducibility and accurate quantitation[1,2]; gas chromatography provides the latter advantages but cannot be used with the thermally labile carotenoids[3,4].

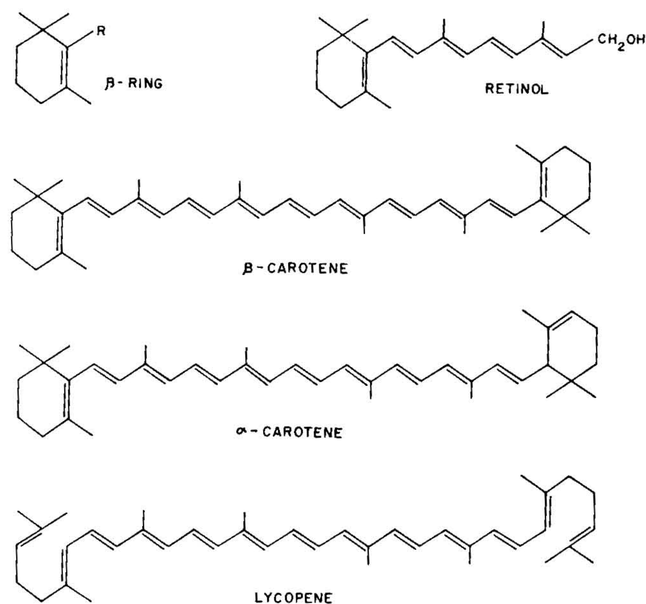


Fig. 1. Importance of β-ring in carotenoid activity as vitamin A precursors.

The advent of high-performance liquid chromatography (HPLC), which made available rapid, reproducible, quantitative and accurate analyses, opened up new possibilities for the study of carotenoids. The use of this technique together with the recently developed microparticle packings has resulted in faster solute distributions between the mobile and stationary phases, hence sharper elution profiles for individual compounds and the increased resolution capacity for a given separation[5].

Although some carotenes could not be separated by HPLC on silica [6,7], a carotenoid mixture was resolved on both magnesium oxide[1] and alumina columns[8] by gradient elution; however, long re-equilibration periods at initial conditions were necessary for subsequent analysis[8]. The isolation of some isoprenoids and polyprenols was also successful on chemically-bonded, reversed-phase packings[9,10]. These non-

polar stationary phases present several advantages over the normal-mode polar adbents: they are neutral to the sample and unaffected by the presence of water or changes in the mobile phase [9,11]. This inherent stability renders them most suitable for routine sample analysis. The separation of carotenes was thus attempted by the use of organic eluting solvents in reversed-phase, and the resolution of β-carotene from other carotenoids was sought for estimation of provitamin A values in tomato samples.

MATERIALS AND METHODS

Standard solutions

All standards were purchased from Sigma (St. Louis, Mo., U.S.A.). Crystalline α-carotene was dissolved in petroleum ether; the resulting solution contained 333.75 μg/ml (0.5216 mmoles/l). Crystalline β-carotene was dissolved in petroleum ether (b.p., 40–60°) the resulting solution contained 154 μg/ml (0.2869 mmoles/l). The concentration of lycopene dissolved in dichloromethane was 166.66 μg/ml (0.3104 mmoles/l, solution I); 1 ml of this solution in 25 ml dichloromethane constituted solution II (0.0124 mmoles/l).

The retention times of lycopene, α- and β-carotene averaged 7.82, 12.4 and 13.2 min, respectively. The peak areas were measured for quantitation.

Liquid chromatograph

A Waters Model 6000A Solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) was used. This is a reciprocating plunger pump which permits digital selection of a constant flow-rate. Waters U6K injector system allowed loading of the sample at atmospheric pressure and assured accurate injections (no sample loss due to back pressure on the system). A Waters Model 450 Variable Wavelength Detector was set at 470 nm. This wavelength was found most suitable for the simultaneous detection of α-carotene, β-carotene and lycopene.

A SF 770 Spectroflow Monitor variable-wavelength detector equipped with SFA 339 Wavelength drive and MM 700 Memory Module, all from Kratos Inc., Schoeffel Instrument Division (Westwood, N.J., U.S.A.), was used for obtaining stopped-flow visible spectra.

Chromatographic peaks were recorded on a Houston Omniscribe recorder. The following chromatographic columns were used in the course of this study: stainless-steel (30 cm × 3.9 mm I.D.) μ-Porasil (Waters); stainless-steel (25 cm × 4.6 mm I.D.) packed with LiChrosorb RP-8 (Brownlee, Santa Clara, Calif., U.S.A.); stainless-steel (30 cm × 3.2 mm I.D. μBondapak C_{18} (Waters); stainless-steel (25 cm × 4.6 mm I.D.) Partisil-PXS-10/25ODS-2 (Whatman, Clifton, N.J., U.S.A.); stainless-steel (25 cm × 4.6 mm I.D.) Partisil-PXS-5/ODS (Whatman).

Solvents

Isooctane, chloroform and acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), diethyl ether (Mallinckrodt, St. Louis, Mo., U.S.A.) and methanol (Matheson, Coldeman & Bell, Norwood, Ohio, U.S.A.) were all residue-free and suitable for chromatography and spectrophotometry. Solvents were filtered through a 0.5-μm glassfiber filter (Gelman, Ann Arbor, Mich., U.S.A.) and degassed under vacuum prior to use.

Preparation of food samples

Extraction procedure. Six tomato samples (red-ripe Massachusetts greenhouse tomatoes), each weighing *ca.* 160 g, were individually cut into small pieces and homogenized under a stream of nitrogen in acetone for 1–2 min in a blender. The initial homogenate was filtered through a sintered glass funnel, pore size 20–30 μm (Whatman), under reduced pressure and the residue recovered for extraction. The procedure was repeated until complete extraction of all pigments was achieved. The acetone extract was then added to an equal volume of freshly distilled, peroxide-free, petroleum ether (PE) in a separatory funnel, mixed and diluted with water. Upon formation of two layers, the lower aqueous phase was re-extracted once with PE and the bulked PE solutions washed three times with water to remove acetone[3,4].

Saponification

Saponification is generally necessary in carotenoid analysis to remove unwanted lipid material which could interfere with the chromatography of compounds of interest.

Extracts were evaporated to dryness using a rotary evaporator, and a solution of 15% KOH in methanol was added to the round bottom flask. The alkaline mixtures were left in the dark for 14 h at room temperature. They were afterwards gradually added to freshly distilled petroleum ether in a separatory funnel. Water was slowly poured into the funnel so as not to form an emulsion. When two phases appeared, the lower aqueous phase was drawn off and extracted three times with fresh volumes of PE. The ethereal solutions were then bulked in a separatory funnel and washed free from alkali by repeated additions of water, followed by discarding the resultant aqueous layers. Each saponified extract was then concentrated to 100 ml in a rotary evaporator and stored under nitrogen in a volumetric flask[3,4].

Removal of sterols

The different samples were kept in the freezer overnight, at $-10°$, and the sterols precipitated to the bottom of the containers[3,4].

Aliquots for HPLC

Ten ml from each flask were filtered through a 0.5-μm glass-fiber filter before injection. Care was always taken not to expose any of the samples to light. All samples were stored under nitrogen in the freezer. Both 50-μl and 10-μl samples in petroleum ether were injected, the former to determine mainly β-carotene amounts and the latter, lycopene. Concentrations of each compound were determined from the slope of the calibration plots in which peak area was plotted against amount injected (nmoles β-carotene or lycopene). The detector responses were found to be linear over the entire working range.

Identification of peaks in HPLC eluents

Initial peak identification was based on retention times and comparison with the standards as well as co-chromatography with the standards. Since retention times alone are not sufficient for positive identification, stopped-flow visible spectra of the chromatographic peaks were also obtained. The Schoeffel variable wavelength detector is equipped with a memory module which automatically stores the spectral background

caused by changes in the optical properties of solvents, flow-cell light path and monochromator, and later subtracts it from the scans of the compounds. In order to obtain a stopped-flow scan, the flow is arrested at the top of each peak and the corrected spectrum scanned over the desired wavelength range.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A Waters μPorasil (10-μm silica particles) was first tried. Pure isooctane, used as a mobile phase, resulted in long retention times (large k' values) and did not resolve α- and β-carotene (*cf.*, ref. 7); with addition of chloroform to isooctane both β-carotene and lycopene were eluted with the same retention time. Gradients were found to be impractical since the column re-equilibration necessitated more than 3 h under any set of conditions.

All other columns used had non-polar packings and were operated under reversed-phase conditions. The Brownlee column packed with LiChrosorb RP-8 (C_8 chemically bonded to silica, 10-μm particle size) resulted in too rapid elutions.

The increased "thickness" of the stationary phase in the C_{18}- over the C_8-coated packings is expected to increase the k' values[12]. This was indeed found to be true with Waters μBondapak C_{18} (10-μm particle size) and Whatman Partisil-PXS-10/25ODS-2 (also 10-μm particle size). The latter column and a mixture of 8.3% chloroform in acetonitrile as a mobile phase gave the best separation for a mixture of α- and β-carotene standards, although resolution (R_s = 0.63) was still inadequate.

TABLE I

INJECTIONS OF β-CAROTENE, α-CAROTENE AND LYCOPENE STANDARDS ON PARTISIL-10/ODS-2 AND PARTISIL-5/ODS

	Partisil-10/ODS-2		*Partisil-5/ODS*		
	Chloroform in acetonitrile		*Chloroform in acetonitrile*		
	11.6% (1 ml/min)	*8.5% (1 ml/min)*	*11.6% (2 ml/min)*	*8.5% (2 ml/min)*	*8.0% (2 ml/min)*
k'					
β-Carotene	6.0	8.48	5.84	7.90	8.84
α-Carotene	5.57	7.62	5.56	7.43	7.92
Lycopene	3.23	4.63	3.21	4.31	4.84
α					
$\frac{k'_{\beta\text{-Car}}}{k'_{\alpha\text{-Car}}}$	1.07	1.11	1.05	1.06	1.11
$\frac{k'_{\alpha\text{-Car}}}{k'_{\text{Lyc}}}$	1.72	1.64	1.73	1.72	1.63
$\frac{k'_{\beta\text{-Car}}}{k'_{\text{Lyc}}}$	1.85	1.83	1.82	1.83	1.82
$R_s = \frac{2(V_{\beta\text{-Car}} - V_{\alpha\text{-Car}})}{(W_{\beta\text{-Car}} + W_{\alpha\text{-Car}})}$		0.63	1.0	1.2	>1.5

Increasing the efficiency of the column by using 5-μm particles[13] in Whatman Partisil-PXS-5/ODS improved the resolution of the α- and β-carotene standards to a value of 1.2 (Table I). Samples were then chromatographed using this column and a mobile phase containing 8% chloroform in acetonitrile. Fig. 2 shows a chromatogram resulting from injecting the three standards simultaneously: the geometric isomers, α- and β-carotene, were resolved.

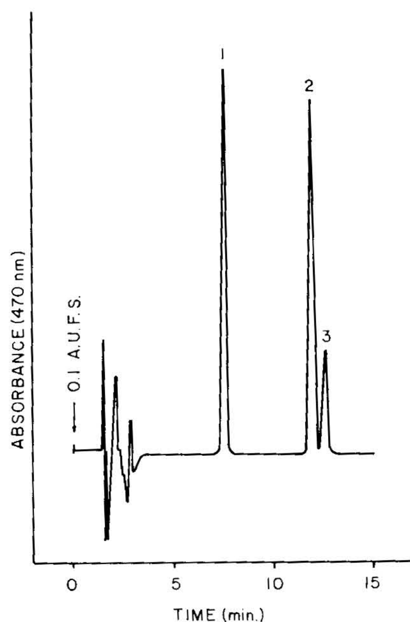


Fig. 2. Separation of standards: 1 = lycopene; 2 = α-carotene; 3 = β-carotene (R_s for peaks no. 2 and 3, 1.46). Chromatographic conditions: column, Partisil-5/ODS, 5 μm; eluent, 8.0% chloroform in acetonitrile; flow-rate, 2.0 ml/min; temperature, ambient; detection, 470 nm; sensitivity, 0.1 A.U.F.S.

Peak identification and quantitation

Under the chromatographic conditions used, the lower detection limits for lycopene, α-carotene and β-carotene standards were found to be 0.00395, 0.0372 and 0.0285 nmoles, respectively.

Chromatograms of tomato extracts run under the conditions described in the experiment are shown in Fig. 3. In reversed-phase chromatography, more polar compounds elute first[14]. This, together with classical literature on tomato pigments[15,17], indicates that the early eluted peak, X is probably a xanthophyll whereas peak Y, which elutes between lycopene and β-carotene, might be γ-carotene. α-Carotene is not prominent in any sample. As expected[15], the major components of tomato extracts, lycopene and β-carotene, were found to be in a *ca.* 9:1 ratio. Identification of these was done by comparing both the retention times and the visible spectra (380–600 nm) of the peaks from the extracts and the standard solutions. Close agreement between electronic absorption spectra (stopped-flow scanning method) confirms this identifi-

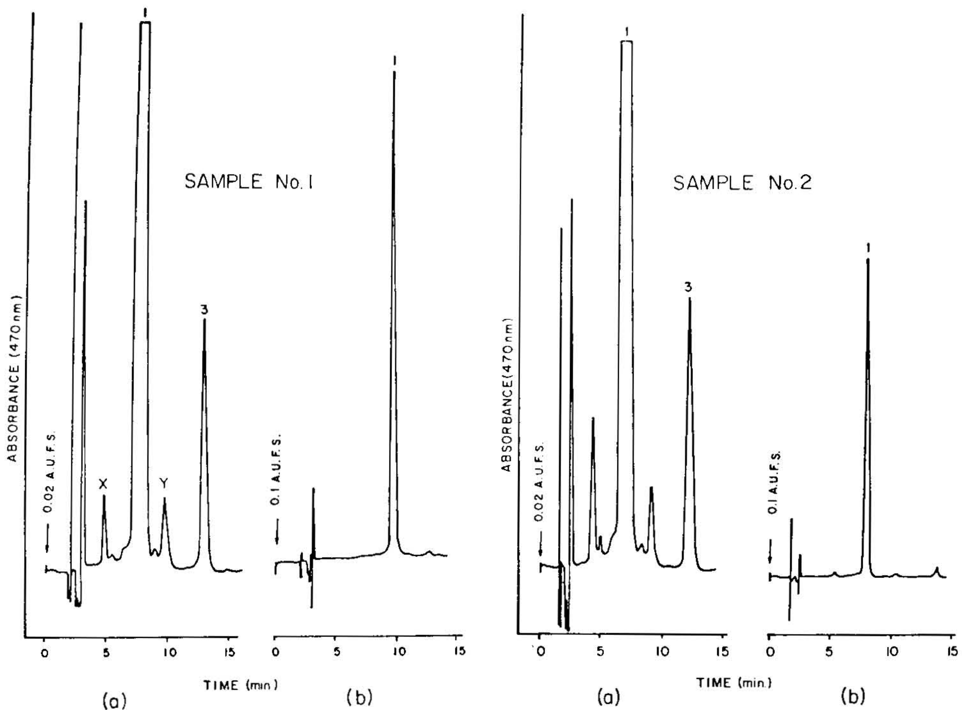


Fig. 3. Chromatograms of two tomato extracts: (a) 50 μl injected; (b) 10 μl injected. Chromatographic conditions as in Fig. 2. Peaks 1 and 3 are lycopene and β-carotene, respectively.

cation since the absorbance is a function of the chromophore[3], thus a characteristic of each carotenoid (Fig. 4).

The chromatographic peaks of β-carotene and lycopene were quantitated using the external calibration method; the values obtained for the tomato samples studied ware shown in Table II.

A report from the National Academy of Sciences estimates that only one sixth of the β-carotene from a diet is actually converted to retinol in humans[18]. The average β-carotene value of five samples, excluding sample No. 5, amounted to 1.218 μg/g or 1.218/6 = 0.2031 μg/g retinol equivalents. Since 0.3 μg retinol is by definition equivalent to 1 International Unit (I.U.), of vitamin A, the tomatoes studied contained 0.2031/0.3 = 0.6771 I.U./g.

The actual potency of the sample may be a little underestimated since γ-carotene, also a vitamin A precursor[16], was not accounted for. The resulting differences, however, should not be too important since γ-carotene is known to occur only in very small quantities in tomatoes[15,17] and has only one half the bioactivity of β-carotene[16].

On the other hand, including inactive pigments such as lycopene, which is present in large amounts in the tomato fruit, would overrate the provitamin A content of the sample. The method of the Association of Official Analytical Chemists (AOAC) which includes this carotenoid as well as others[8], results in erroneously high values (Table III).

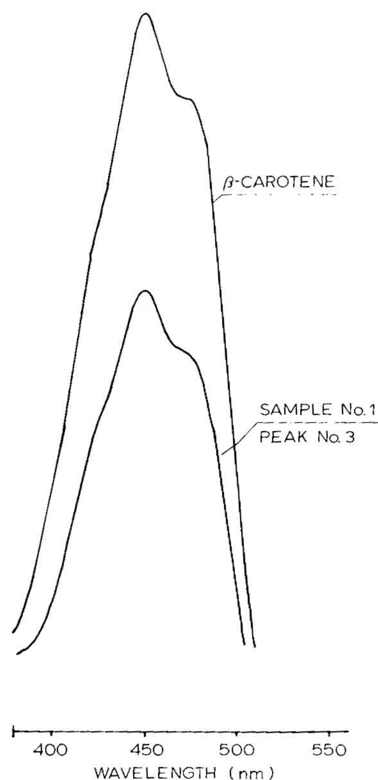


Fig. 4. Comparison of visible absorption spectra of β-carotene standard and peak no. 3 in sample no. 1. Scanning rate, 100 μm/min; sensitivity, 0.02 A.U.F.S.

TABLE II

QUANTITATION OF LYCOPENE AND β-CAROTENE IN TOMATO SAMPLES

Sample No.	*Lycopene (μg/g sample)*	*β-Carotene (μg/g sample)*
1	3.840	1.196
2	9.926	1.209
3	8.981	1.270
4	9.985	1.205
5	9.295	0.805
6	10.72	1.212

TABLE III

MEASUREMENT OF PROVITAMIN A VALUES IN TOMATOES: COMPARISON OF THE HPLC AND AOAC METHODS

In each case it is assumed that one sixth of the measured pigment(s) is converted to retinol.

Methodology	*Av. value (μg/g)*	*Retinol equivalent (μg/g)*	*I.U./g*
β-Carotene as obtained by HPLC	1.218	0.203	0.677
β-Carotene and lycopene obtained by HPLC	11.001	1.833	6.111
AOAC method	18.063	3.010	10.035

It is therefore of interest to quantitate vitamin A precursors only and to improve literature values which were obtained by using classical chromatographic and spectrophotometric techniques.

In summary, a new HPLC reversed-phase method was developed for the separation of carotenoids using a 5-μm particle column. The analysis of the saponified and washed extract is performed isocratically in less than 15 min. With the mobile phase of 8% chloroform in acetonitrile, the column pressure was found to be 2000 p.s.i.; it however, increased beyond usable range over period of time. The newly developed 5-μm packings, although more efficient than the regular 10-μm particles, have a shorter column life.

Repeated injections of standards and sample No. 1 were used to demonstrate the reproducibility of peak areas and retention times. The reversed-phase packings possess great stability and the ability to separate isocratically compounds of a wide polarity range (the acyclic lycopene being different from the bicyclic double-bond positional isomers *α*- and *β*-carotene).

In addition, nanogram quantities of compounds under study can be detected and since the analysis time is short the pigment decomposition and formation of artifacts are minimized[1]. All this makes the described HPLC system suitable for routine assays of provitamin A content in natural products. This is only a preliminary study and more work is necessary to insure the significance of quantitative data. This method can easily be applied to the analyses of various other vegetables and fruits, in order to determine the best natural sources of provitamin A, encourage their growth and promote their daily consumption.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the contribution of Dr. Richard Hartwick in the course of this project.

REFERENCES

1 I. Stewart, *J. Agr. Food Chem.*, 25 (1977) 1132.
2 T. Hiyarna, M. Nishimura and B. Chance, *Anal. Chem.*, 29 (1969) 339.
3 B. H. Davies, in T. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Academic Press, New York, 2nd ed., 1976, pp. 38–165.
4 L. C. Raymundo, A. E. Griffiths and K. L. Simpson, *Phytochemistry*, 6 (1967) 1527.
5 C. A. Frolik, T. E. Tavela and M. B. Sporn, *J. Lipid Res.*, 19 (1978) 32.
6 I. Stewart, *J. Assoc. Offic. Anal. Chem.*, 60 (1977) 132.
7 A. Fiksdahl, J. T. Mortensen and S. Liaaen-Jensen, *J. Chromatogr.*, 157 (1978) 111.
8 S. K. Reeder and G. L. Park, *J. Assoc. Offic. Anal. Chem.*, 58 (1975) 595.
9 P. C. Donnahey and F. N. Heming, *Biochem. Soc. Trans. 557th Meeting, Liverpool, 1975*, Vol. 3, pp. 775–776.
10 T. Chojnacki, W. Jankowski, T. Mankowski and W. Sasak, *Anal. Biochem.*, 69 (1975) 114.
11 K. Eskins, C. R. Scholfield and H. J. Dutton, *J. Chromatogr.*, 135 (1977) 217.
12 A. Nakae and G. Muto, *J. Chromatogr.*, 120 (1976) 47.
13 S. K. Hajibrahim, P.J. C. Tibbetts, C. D. Watts, J. R. Maxwell, G. Eglinton, H. Colin and G. Guiochon, *Anal. Chem.*, 50 (1978) 549.
14 C. G. Simpson, *Practical High Performance Liquid Chromatography*, Hyden & Son, New York, 1976, pp. 8–12.
15 J. C. Bauernfeind, *Encyclopedia of Food Technology and Food Science Series*, 3 (1977) 113.
16 B. Borenstein and R. H. Bunnel, *Advan. Food Res.*, 15 (1967) 195.
17 J. L. Fiasson, N. Arpin, P. Lebreton and P. Bouchez, *Chim. Anal. (Paris)*, 51 (1969) 227.
18 *Recommended Dietary Allowances*, National Academy of Sciences, Washington, D.C., 8th ed., 1974, pp. 50–54.

Journal of Chromatography, 176 (1979) 118–122
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,864

Note

Gas chromatographic determination of low-molecular-weight carbonyl compounds in aqueous solution as their pentafluorophenylhydrazones

KEIKO KOBAYASHI, MICHIRU TANAKA, SATOSHI KAWAI and TAKEO OHNO
Gifu College of Pharmacy, Mitahora Higashi, Gifu (Japan)
(Received February 23rd, 1979)

Previously, we reported that the photolysis of aliphatic α-amino acids in the presence of mercury(II) chloride resulted in the formation of methylmercury, arising from apparent fragmentation of the alkyl residue of the α-amino acids[1].

Recently, it has been found that photochemical alkylation of inorganic mercury proceeds in a more complicated manner in neutral media[2]. In considering the mechanism of the alkylation, we were confronted with the problem that it was necessary to measure quantitatively the low-molecular-weight carbonyl compounds produced as intermediates in the photolysis of α-amino acids. Low-molecular-weight carbonyl compounds are known to be extremely soluble in water. The reaction between carbonyl compounds and 2,4-dinitrophenylhydrazine is known to be an effective derivatization process which yields compounds that are extractable with organic solvents.

On the other hand, gas chromatography (GC) requires the previous preparation of suitable volatile derivatives. Attal *et al.*[3] and Mead *et al.*[4] reported on the GC of carbonyl compounds using pentafluorophenylhydrazine (PFPH). This method has been applied to lower aliphatic carbonyl compounds by Hoshika and Muto[5]. However, they showed only typical chromatograms for standard solution of pure pentafluorophenylhydrazones which were prepared in methanol solution, and we are not aware of any report dealing with an aqueous solution containing lower aliphatic carbonyl compounds. This paper describes a procedure for the GC determination of low-molecular-weight carbonyl compounds in aqueous solution as their pentafluorophenylhydrazones and its application to such compounds produced by photolysis of α-amino acids.

EXPERIMENTAL

Reagents

Pentafluorophenylhydrazine (PFPH) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). The buffer solution was 0.6 *M* sodium phosphate solution (pH 7.0). The internal standard (IS) solution was a 0.025% solution of *p*-xylylene dichloride in ethyl acetate.

Apparatus and conditions

A Shimadzu GC-4APF gas chromatograph equipped with a flame-ionization detector (FID) was used. The GC conditions were as follows: a 2-m glass column packed with 3% XE-60 on 80–100-mesh Celite 545 (AW DMCS), column temperature 120°, detector temperature 150°, injection temperature 150° and chart speed 0.25 cm/min.

Standard procedure

To 0.5 ml of sample solution, add 0.5 ml of PFPH solution (1.5 mg/ml; *ca.* $7.6 \cdot 10^{-3}$ *M*) and 1 drop of 0.6 *M* phosphate buffer (pH 7.0) in a 10-ml centrifuge tube. Mix well and allow to stand for 20 min at room temperature. Saturate the reaction mixture with sodium chloride, add 1 drop of 18 *N* sulphuric acid and extract with 0.2 ml of ethyl acetate containing 50 μg of *p*-xylylene dichloride as internal standard. Carry out the extraction in the cold. Remove excess of sodium chloride and the aqueous layer with the aid of a syringe with a long needle, dry by adding a small amount of sodium sulphate and apply an aliquot of the ethyl acetate extract on to the GC column.

RESULTS AND DISCUSSION

The relative retention times of twelve pentafluorophenylhydrazone derivatives and some compounds that are possible internal standards are given in Table I.

TABLE I

RELATIVE RETENTION TIMES OF THE PENTAFLUOROPHENYLHYDRAZONES OF TWELVE CARBONYL COMPOUNDS AND SOME COMPOUNDS THAT ARE POSSIBLE INTERNAL STANDARDS

Parent compounds	*Stationary phase and column temperature*			
	2% OV-17, 100°	*3% SE-30, 100°*	*3% XE-60, 120°*	*3% XF-1105, 115°*
PFPH	0.67	0.19	0.44	0.25
HCHO	0.33	0.22	0.51	0.34
CH_3CHO	0.56	0.43*	0.61*	0.49
C_2H_5CHO	0.94	0.77	0.93	0.80
n-C_3H_7CHO	1.48	1.32	1.44	1.31
iso-C_3H_7CHO	1.00	1.00	1.00	1.00
n-C_4H_9CHO	2.78	2.81	2.34	2.50
CH_3COCH_3	0.69	0.68	0.69	0.65
$CH_3COC_2H_5$	1.30	1.15	0.97	1.03
CH_3CO-*iso*-C_3H_7	1.37	1.55	1.10	1.32
CH_3CO-*iso*-C_4H_9	2.32	2.48	1.73	2.05
$C_2H_5COC_2H_5$	1.70	1.81	1.22	1.50
C_2H_5CO-*n*-C_3H_7	2.58	2.90*	1.64*	2.20
I-C_6H_4-I	2.98	1.34	1.11	1.14
Br-C_6H_4-Br	0.52	0.54	0.30	0.34
Cl-C_6H_4-Cl	0.61	0.39	0.39	0.34
Cl-C_6H_4-CH_2Cl	0.64	0.48	0.43	0.38
$ClCH_2$-C_6H_4-CH_2Cl	2.39	1.50	1.56	1.11

* Double peaks.

Hoshika and Muto[5] reported that the separation of the peaks of PFPH and the formaldehyde pentafluorophenylhydrazone derivative was incomplete when either 5% SE-30 or a glass capillary coated with DEG 20M was used. As shown in Table I, however, the separation of the both of these peaks was adequate for the determination of formaldehyde on any of the columns we used. Fig. 1 illustrates a typical separation of some carbonyl compounds as their pentafluorophenylhydrazones using a 3% XE-60 column. *p*-Xylylene dichloride was used as the internal standard. A series of preliminary investigations was carried out in order to find suitable conditions for reaction and extraction, and the procedure described under Experimental was established. The condensation reaction proceeded more readily in neutral than in acidic media.

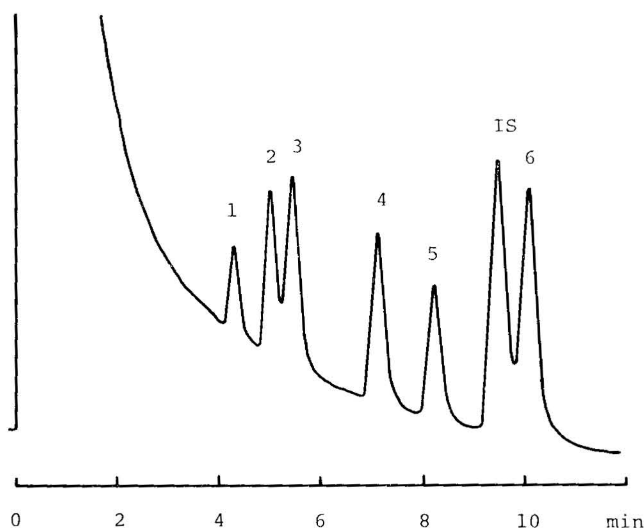


Fig. 1. Gas chromatogram of some carbonyl compounds as their pentafluorophenylhydrazones. Conditions: 3% XE-60, 2.0-m glass column, temperature programmed from 105° to 130° at 2°/min, FID. Peaks: 1 = formaldehyde; 2 = acetaldehyde; 3 = acetone; 4 = isobutylaldehyde; 5 = diethyl ketone; IS = *p*-xylylene dichloride; 6 = methyl isobutyl ketone.

Table II shows the effect of the reaction period at room temperature on the extent of the condensation reaction with 0.5 ml of a $7 \cdot 10^{-4}$ *M* solution of each carbonyl compound according to the procedure described. In general, aldehydes readily underwent quantitative reactions in 20 min, while the reactions with ketones proceeded slowly and showed a tendency to become even slower as the alkyl residue became larger. It seems likely that the lowering of the extent of reaction is due to steric hindrance of bulky, alkyl groups.

The reagent concentration was made about 10 times greater than those of the carbonyl compounds and the reaction period for aldehydes was fixed at 20 min at room temperature in order to obtain constant extents of reaction.

Of the carbonyl compounds tested, some gave double peaks on the chromatograms, possibly due to *syn-* and *anti-*isomers resulting from condensation reactions with PFPH.

TABLE II

EFFECT OF REACTION PERIOD ON EXTENT OF CONDENSATION REACTION WITH PFPH

Compound	*Reaction period (min)* *				
	0	*20*	*40*	*120*	*24 h*
HCHO	0.37**	0.58	0.57	0.52	0.54
CH_3CHO	0.33	0.67	0.67	0.62	0.64
C_2H_5CHO	0.60	0.62	0.64	0.65	0.68
n-C_3H_7CHO	0.49	0.46	0.51	0.54	0.53
iso-C_3H_7CHO	0.55	0.82	0.81	0.81	0.80
n-C_4H_9CHO	0.97	1.00	1.03	1.14	1.11
CH_3COCH_3	0.08	0.25	0.29	0.72	0.76
$CH_3COC_2H_5$	0.06	0.10	0.18	0.41	0.78
CH_3CO-*iso*-C_3H_7	0.02	0.07	0.08	0.19	0.83
CH_3CO-*iso*-C_4H_9	0.01	0.04	0.06	0.13	0.38
$C_2H_5COC_2H_5$	0.02	0.05	0.07	0.12	0.30
C_2H_5CO-*n*-C_3H_7		0.04	0.06	0.08	0.22

* At room temperature.

** The extent of the reaction was determined as the peak-area ratio of the compound peak to that of the internal standard (*p*-xylylene dichloride).

Ethyl acetate was a suitable solvent for the extraction of the hydrazone reaction products. Salting-out improved the extent of extraction. Extraction was carried out in acidic media to prevent an excess of PFPH from being extracted into the organic solvent together with the reaction hydrazone products. It would also be desirable to carry out extraction at reduced temperatures and dehydration by adding a small amount of anhydrous sodium sulphate. Standard solutions of pure hydrazones in ethyl acetate were stable.

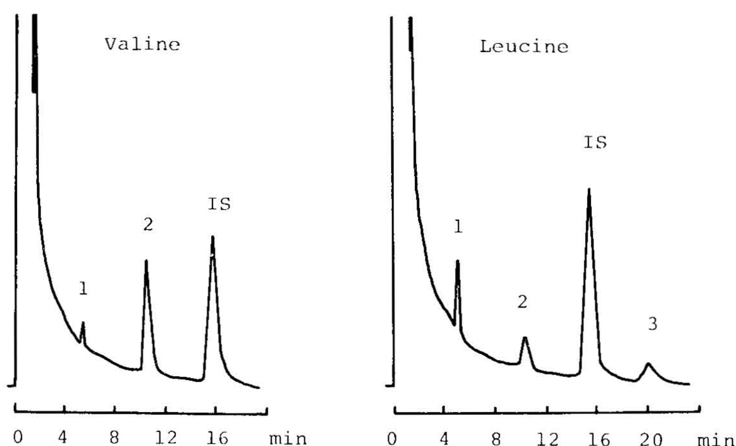


Fig. 2. Gas chromatograms of pentafluorophenylhydrazones of some carbonyl compounds produced from photochemical reactions of amino acids. Conditions: 3% XE-60, 2.0-m glass column, 120°, FID. Peaks: 1 = formaldehyde; 2 = acetaldehyde; IS = xylylene dichloride; 3 = isovaleraldehyde (?).

Sample solutions containing three carbonyl compounds (formaldehyde, acetaldehyde and isobutyraldehyde) were measured according to the procedure described, and the calibration graph for each was linear and passed through the origin for concentrations in the range 10–40 μg. Five repeated determinations on an identical sample solution containing 30 μg of isobutyraldehyde gave a standard deviation of 1.84%.

Solutions of DL-alanine, DL-valine, DL-leucine or DL-isoleucine (7.5 mmole) in 0.1 M phosphate buffer (pH 7.0) (300 ml) containing mercury(II) chloride (2.5 mmole) were irradiated in a quartz vessel with a 20-W blacklight lamp (300–400 nm) as a light source for 300 h and the mixtures were measured by the method described.

Fig. 2 shows the gas chromatograms obtained. For valine, two peaks corresponding to formaldehyde and isoburaldehyde were observed. For leucine, a peak thought to be isovaleraldehyde from its retention time, in addition to isobutyraldehyde and formaldehyde, was observed.

REFERENCES

1 K. Hayashi, S. Kawai, T. Ohno and Y. Maki, *Chem. Commun.*, (1977) 158.
2 K. Kobayashi, S. Kawai and Y. Maki, unpublished results.
3 J. Attal, S. M. Hendeles and K. B. Eik-Nes, *Anal. Biochem.*, 20 (1967) 394.
4 R. A. Mead, G. C. Haltmeyer and K. B. Eik-Nes, *J. Chromatogr. Sci.*, 7 (1969) 554.
5 Y. Hoshika and G. Muto, *J. Chromatogr.*, 152 (1978) 224.

Journal of Chromatography, 176 (1979) 123–125
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,867

Note

Gas chromatographic determination of preservatives in rennet

A. GEAHCHAN, M. PIERSON and P. CHAMBON
Laboratoire de Toxicologie, Faculté de Pharmacie de Lyon, 8 Avenue Rockefeller, 69008 Lyon (France)
(Received March 1st, 1979)

Sorbic acid and benzoic acid are frequently added to food products as preservatives to prevent the development of moulds and bacteria. We have tried to develop a rapid and accurate procedure for evaluating these two preservatives in rennet, using gas chromatography. Already published methods relied either on direct injection[1–5] or indirect injection, either after silylation[6] or methylation with methanol–hydrochloric acid[7], methanol–boron trifluoride[8] or diazomethane[9–11]. We found that the best results were obtained by conversion into methyl esters with diazomethane, and by adding an internal standard (undecanoic acid)[1].

Prior to the gas chromatographic analysis, the acids were isolated by extraction of the rennet sample with diethyl ether in acidic media.

EXPERIMENTAL

Apparatus

A gas chromatograph (Fractovap 2350, Carlo Erba, Milan, Italy) with a flame-ionization detector was used. The carrier gas was nitrogen at a flow-rate of 30 ml/min. Glass columns (2 m × 2 mm I.D.) were packed with 15% EGA coated on 80–100-mesh Chromosorb W AW DMCS. The column temperature was 130° and the injector temperature 250°. A Hewlett-Packard 3380A recorder–integrator was employed.

Reagents

Analytical-reagent grade benzoic, sorbic and undecanoic acid (Fluka, Buchs, Switzerland) were used as standards. Standard solutions of 1 g/l of benzoic and sorbic acid in diethyl ether were prepared. The internal standard solution was a 1 g/l solution of undecanoic acid in diethyl ether.

Analytical-reagent grade methanol and diethyl ether were employed, and analytical-reagent grade concentrated hydrochloric acid was used to prepare a 0.1 *N* solution.

The methylation reagent (diazomethane) was prepared by adding to 1 g of N-nitrosotoluene-4-sulphomethylamide (Fluka) 30 ml of diethyl ether, 6 ml of methanol and 10 ml of 6% potassium hydroxide solution. The supernatant fraction was introduced into a twonecked flask from which diazomethane was transferred into a second flask using nitrogen as carrier gas.

Preparation of methylated standards

To 1 ml each of the benzoic and sorbic acid solutions are added 1 ml of the undecanoic acid internal standard solution and a mixture of 0.3 ml of methanol and 1 ml of diethyl ether. Methylation is then effected by treatment with gaseous diazomethane until the development of a yellow colour. The final volume is made up to 10 ml with diethyl ether. A 1-μl volume of the solution corresponds to 100-ng aliquots of benzoic, sorbic and undecanoic acid.

Isolation and methylation of the preservatives

To 0.5 ml of rennet in a ground-glass stoppered test-tube are added 2 ml of 0.1 *N* hydrochloric acid and the mixture is extracted three times with 5 ml of diethyl ether with vigorous agitation. The ethereal layers are concentrated to approximately 3 ml by heating at 30° under a flow of nitrogen. A 1-ml aliquot of the undecanoic acid internal standard solution and 0.3 ml of methanol are added. Methylation of the sample is effected as described for the standards, followed by injection of 1 μl into the gas chromatograph.

RESULTS AND DISCUSSION

Peak areas were calculated for benzoic acid and sorbic acid, and the relative response for each preservative was compared with that of the internal standard (undecanoic acid) by means of the integrator.

Fig. 1 illustrates the good separation of the methylated acids, with retention

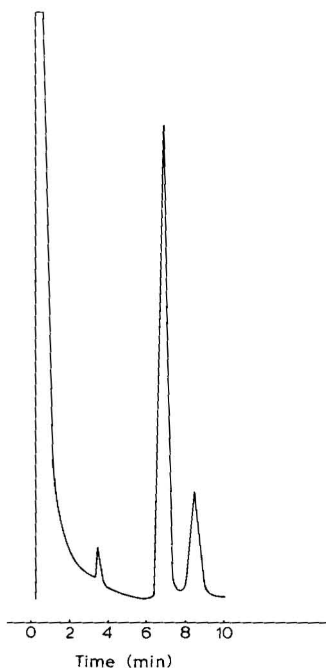


Fig. 1. Gas chromatogram of methyl esters of sorbic acid (retention time 3.52 min), benzoic acid (6.99 min) and undecanoic acid (8.56 min). Each peak is equivalent to 100 ng.

times of 3.52 min for sorbic acid, 6.99 min for benzoic acid and 8.56 min for undecanoic acid.

The smallest amount of preservative detectable is 20 ng/μl in the actual extract, equivalent to 400 mg/l in the rennet sample. The recovery was checked by extraction of known amounts of preservatives from aqueous solutions according to the procedure previously described, followed by UV spectrometry. It was found to be above 95% for concentrations as low as 20 mg/l.

Hence this method permits the specific and rapid (about 30 min) quantitative determination of benzoic and sorbic acid in rennet, with very satisfactory recoveries.

REFERENCES

1 A. Bertrand and Ch. Sarre, *Ann. Fals. Expert. Chim.*, 71 (1978) 35.
2 E. G. C. Clarke, D. J. Humphreys and E. Stoilis, *Analyst (London)*, 97 (1972) 433.
3 E. Fogden, M. Fryer and S. Urry, *J. Ass. Publ. Anal.*, 12 (1974) 93; *Anal. Abstr.*, 28 (1975) 6F18.
4 A. Graveland, *J. Ass. Offic. Anal. Chem.*, 55 (1972) 1024.
5 M. Toyoda, T. Kanamori, I. Yosho and M. Iwaida, *Eisei Kagaku*, 23 (1977) 100; *Anal. Abstr.*, 33 (1977) 5F24.
6 J. A. W. Gosselé, *J. Chromatogr.*, 63 (1971) 429.
7 D. Revuelta, G. Revuelta and F. Armisen, *An. Quim.*, 71 (1975) 179; *Anal. Abstr.*, 29 (1975) 4F28.
8 A. Sasson, Y. Erner and S. P. Monselise, *J. Agr. Food Chem.*, 24 (1976) 652.
9 J. Flek and V. Sedivec, *Collect. Czech. Chem. Commun.*, 36 (1971) 3108; *Anal. Abstr.*, 22 (1972) 4232.
10 N. N. Mikhailovskaya and N. A. Deineko, *Tr. Gos. Nauchno-Issled. Proektn. Inst. Aztn. Prom. Prod. Org. Sint.*, 13 (1972) 176; *Anal. Abstr.*, 25 (1973) 3153.
11 K. Silberreisen and B. Wagner, *Monatsschr. Brau.*, 23 (1970) 55.

Journal of Chromatography, 176 (1979) 126–128
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,876

Note

Gas chromatographic analysis of 1-monoacylglycerols as cyclic carbonate derivatives

JÖRG OEHLENSCHLÄGER* and GÜNTHER GERCKEN**

Institut für Organische Chemie und Biochemie, Abteilung für Biochemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-2000 Hamburg 13 (G.F.R.)

(First received February 19th, 1979; revised manuscript received March 22nd, 1979)

Diacetates or trimethylsilyl (TMS) ethers are commonly used derivatives for the gas–liquid chromatographic (GLC) or gas–liquid chromatographic–mass spectrometric (GLC–MS) analysis of monoacylglycerols[1]. Though both exhibit excellent gas chromatographic properties they are less suitable for the mass spectrometric identification of molecular species because the mass spectra show either no molecular ion or one of poor intensity[2–5].

During our investigations of the synthesis of enantiomeric diacyl-*sn*-glycerols, we used for protection of the free hydroxy moieties of monoacylglycerols the β,β,β-trichloroethylcarbonate group, which rearranged spontaneously in the presence of pyridine to the cyclic carbonate. These monoacylglycerolcarbonates also seemed to be appropriate for the analysis of molecular species.

In this investigation the 1-monoacylglycerols were separated by GLC as cyclic carbonate derivatives (Fig. 1), a class of compounds which shows a characteristic mass spectrometric fragmentation pattern. The advantage of monoacylglycerol cyclic carbonates for application in mass spectrometry has been shown earlier[6]. The mass spectra show molecular ions and acyl radial cations of great intensity, and the glycerol cyclic carbonate moiety in the molecule is well established by two prominent radial cations at m/e = 160 and m/e = 173, respectively, forming the base peak in almost all spectra.

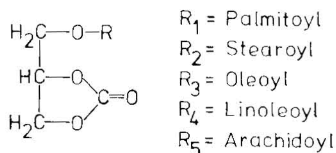


Fig. 1. Structure of *rac*-1-monoacylglycerol-2,3-carbonates.

* Present address: Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Palmaille 9, D-2000 Hamburg 50, G.F.R.

** To whom correspondence should be addressed.

MATERIALS

Enantiomeric glycerol cyclic carbonates were synthesized from naturally occurring precursors such as D-mannitol or D- and L-serine. The cyclic carbonate moiety was introduced into the glycerol backbone by the cyclization of 1-β,β,β-trichloroethylcarbonate glycerol in pyridine at 80°. In an alternative procedure the reaction of 1-O-benzylglycerol with potassium hydrogencarbonate/diethyl carbonate to form 1-O-benzylglycerol-2,3-carbonate was used. The glycerol cyclic carbonates were converted into the corresponding 1-monoacylglycerol-2,3-carbonatees by different acylation methods. The complete synthesis is described elsewhere[6]. All reagents and solvents were from Merck (Darmstadt, G.F.R.). The unsaturated fatty acids and fatty acid chlorides were from Sigma (St. Louis, Mo., U.S.A.) and NU CHEK PREP (Elysian, Minn., U.S.A.).

METHODS

GLC analysis was performed on a Packard Becker Model 419 gas chromatograph (Delft, The Netherlands) equipped with a flame ionization detector (FID), a recorder (Kipp & Zonen, Delft, The Netherlands), and a 6-ft. glass column (3 mm I.D.) packed with 10% Silar 5 CP on Gas-Chrom Q (80–100 mesh) (Applied Science Labs., State College, Pa., U.S.A.).

The column was preconditioned at the column oven temperature of 290° for 20 h, and at a constant flow-rate of helium (40 ml/min). Temperatures were maintained at 268° for the column, 270° for the injector, and 300° for the detector. The helium carrier gas flow-rate was 26 ml/min. The FID gas flow-rates were: hydrogen 30 ml/min and air 300 ml/min.

RESULTS AND DISCUSSION

The retention times of the 1-monoacylglycerol-2,3-carbonates are listed in Table I, relative to that of 1-palmitoylglycerol-2,3-carbonate.

TABLE I

RELATIVE RETENTION TIMES OF FIVE 1-MONOACYLGLYCEROL-2,3-CARBONATES

GLC conditions: see under Methods.

Compound	*Relative retention time*
1-Palmitoylglycerol-2,3-carbonate	1.00*
1-Stearoylglycerol-2,3-carbonate	1.58
1-Oleoylglycerol-2,3-carbonate	1.76
1-Linoleoylglycerol-2,3-carbonate	2.02
1-Arachidoylglycerol-2,3-carbonate	2.43

* The true retention time was 32.7 min.

Fig. 2 shows a typical chromatogram of a mixture of 1-monoacylglycerol-2,3-carbonates. All peaks are completely separated within 80 min, and no interfering peaks are found in the range of the peaks of the acylglycerolcarbonates. The 1-mono-

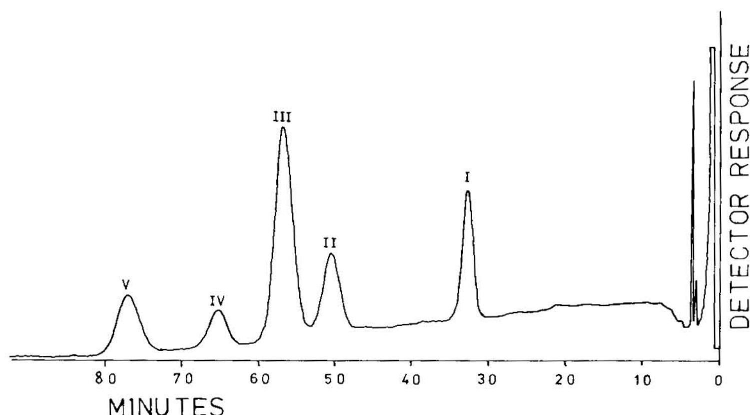


Fig. 2. Gas chromatographic separation of 1-monoacylglycerol-2,3-carbonates on 10% Silar 5 CP on Gas-Chrom Q. Column oven temperature 268°. I: 1-palmitoylglycerol-2,3-carbonate; II: 1-stearoylglycerol-2,3-carbonate; III: 1-oleoylglycerol-2,3-carbonate; IV: 1-linoleoylglycerol-2,3-carbonate; V: 1-arachidoylglycerol-2,3-carbonate.

acylglycerol-2,3-carbonates elute in the same order as found in other fatty acid derivatives, *e.g.* methyl esters[7].

The other derivatives of monoacylglycerols for GLC or GLC–MS, diacetates and TMS ethers, exhibit shorter retention times than the monoacylglycerol cyclic carbonates, but we think that the latter are more appropriate for GLC–MS analysis because of their outstanding mass-spectrometric properties, which permit a simplified detection of molecular species of monoacylglycerols.

CONCLUSION

The GLC separation of 1-monoacylglycerol-2,3-carbonates has been attempted using 10% Silar 5 CP on Gas-Chrom Q. The peaks of the compounds were separated completely within 80 min.

REFERENCES

1 A. Kuksis, in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Marcel Dekker, New York, 2nd ed., 1976, p. 215.
2 C. B. Johnson and R. T. Holman, *Lipids*, 1 (1966) 371.
3 G. Linck, G. Teller and A. Petrovic, *Eur. J. Biochem.*, 58 (1975) 511.
4 J. J. Myher, L. Marai and A. Kuksis, *J. Lipid Res.*, 15 (1974) 382.
5 T. Curstedt, *Biochim. Biophys. Acta*, 360 (1974) 12.
6. J. Oehlenschläger and G. Gercken, *Lipids*, 13 (1978) 557.
7 J. K. Haken, *J. Chromatogr. Sci.*, 13 (1975) 430.

Journal of Chromatography, 176 (1979) 129–133
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

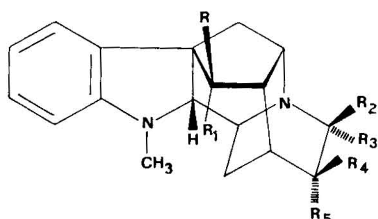
CHROM. 11,865

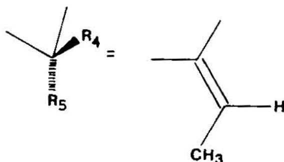
Note

Gas chromatographic determination of ajmaline in the bark of the root of *Rauvolfia vomitoria*

G. P. FORNI
Inverni della Beffa, Laboratorio Ricerche, Via Ripamonti 99, 20141 Milan (Italy)
(Received February 26th, 1979)

Ajmaline is an alkaloid that is widely used as such or as a precursor of semi-synthetic compounds (17-monochloroacetylajmaline*, N-propylajmaline**), in the therapy of arrythmia[1–6]. The most frequently used source of ajmaline is the bark of the root of *Rauvolfia vomitoria*. The industrial process for its production involves its separation from alkaloids of different basicity (reserpine-like compounds) and final purification from alkaloids with extremely similar structures and basicities (isoajmaline sandwichine, etc.).



Ajmaline	$R = R_3 = OH$	$R_1 = R_2 = R_5 = H$	$R_4 = C_2H_5$
Isoajmaline	$R = R_2 = OH$	$R_1 = R_3 = R_4 = H$	$R_5 = C_2H_5$
Sandwichine	$R_1 = R_3 = OH$	$R = R_2 = R_5 = H$	$R_4 = C_2H_5$
Tetraphyllicine	$R = OH$	$R_2 = R_3 = H$	

* Ritmos Elle; Inverni della Beffa, Milan, Italy.
** Neo-Gilurytmal; Kali-Chemie Pharma, Hannover, G.F.R.

The variety of products present creates considerable difficulties for the determination of ajmaline, which is necessary both for the evaluation of drugs in which it is contained and for quality control during its processing.

Only a few methods have been reported for the determination of ajmaline[7–9], and none of them seems suitable for precise, specific and rapid determinations. To resolve this problem, we have utilized a gas chromatographic technique.

EXPERIMENTAL AND RESULTS

Extraction of the drug and purification of the raw extract

Because of the presence of many products in the raw extract of the drug we developed a method for the purification of samples prior to the gas chromatographic analysis. The principle we exploited was the different basicities of the component alkaloids.

A 5-g amount of the drug (coarse powder) is weighed, transferred to a Soxhlet apparatus for continuous extraction and extracted with 150 ml of methanol for 6 h. The extract is evaporated to about 3 ml and transferred to a separator with the aid of 150 ml of 0.01 *M* hydrochloric acid. The solution is extracted with four 50-ml volumes of chloroform, the combined chloroform extracts are washed with three 50-ml volumes of 0.01 *N* hydrochloric acid and the washings are added to the acidic solution.

The chloroform solution is rejected; 1 ml of 85% phosphoric acid is added and the pH of the aqueous solution is adjusted to 8.5 with 5% sodium hydroxide solution. The aqueous solution is extracted with four 70-ml volumes of chloroform and the combined chloroform extracts are dried with anhydrous sodium sulphate and evaporated to dryness under reduced pressure.

Derivatization

Because of the polarity of the compounds, the sample must be silanized before gas chromatographic analysis, according to the following procedure.

The dry extract of the drug, obtained as described above, is dissolved in 250 ml of chloroform, 1 ml of the resulting solution is transferred to a suitable vial containing 1 ml of the internal standard solution (0.05% arbutin in 1:1 chloroform–methanol) and evaporated to dryness with a stream of nitrogen. Then 250 μl of Trisil Z (Pierce, Rockford, Ill., U.S.A.) are added and the vial is sealed.

The sample is heated at 60° for 1 h, cooled to room temperature and 1 μl is injected under the chromatographic conditions described below. The chromatogram obtained is shown in Fig. 1.

Chromatographic system

The analysis is carried out on a Hewlett-Packard Model HP 5830 gas chromatograph equipped with a flame-ionization detector. The carrier gas is pre-purified dry nitrogen at a flow-rate of 20 ml/min; a 2 m × 3 mm I.D. silanized coiled glass column packed with 3% OV-17 on Chromosorb W HP (100–120) mesh is used. The oven temperature is 270°, injection port temperature 280° and detector temperature 300°. Samples are injected with a 10-μl syringe.

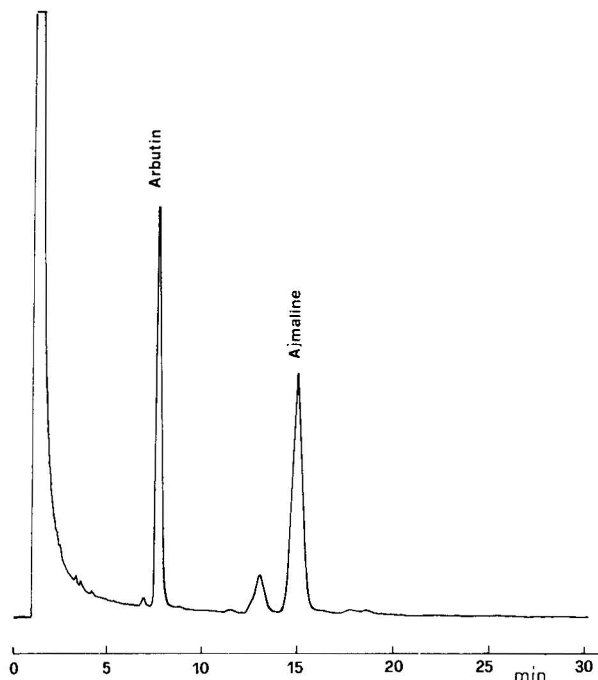


Fig. 1. Gas chromatogram of a sample of *Rauvolfia vomitoria*.

Quantitation

A 1-ml volume of reference solution (0.05% ajmaline in chloroform) is transferred to a suitable vial containing 1 ml of the internal standard solution, evaporated to dryness and silanized as described above. A 1-μl volume is injected.

The calibration data relative to the amount and area of the chromatographic peaks of ajmaline and arbutin are introduced into the integrator, using the internal standard method.

Specificity of the determination

A mixture of isoajmaline, sandwichine, tetraphyllicine, ajmaline and arbutin was analysed under the conditions described. The chromatogram obtained is shown in Fig. 2.

The lack of interference from other products on the chromatographic peak attributed to ajmaline in the drug was also verified by combined gas chromatography–mass spectrometry. The peak was examined by total ion current monitoring; no difference was found in the fragmentation spectrum between pure ajmaline and that analysed in the drug.

Reproducibility and linearity of the chromatographic system

A 100-g amount of ajmaline was analysed ten times following the method described, the results obtained being 99.9, 99.9, 99.6, 102.4, 99.3, 99.1, 98.9, 101.9, 102.0 and 101.5 mg with a mean of 100.45 mg, a standard deviation of 1.35 mg and a

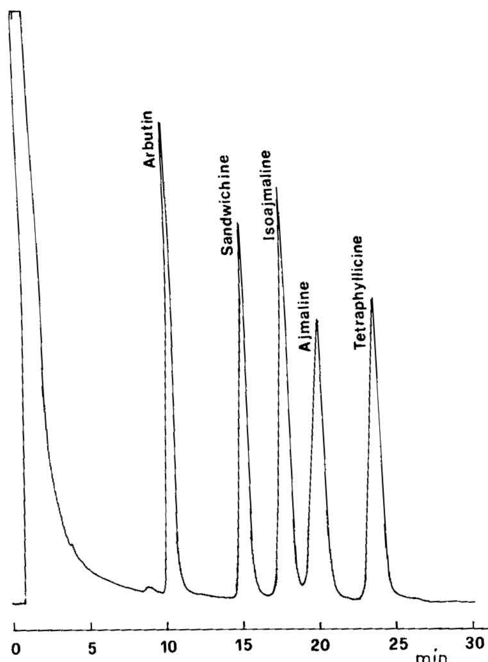


Fig. 2. Gas chromatogram showing the good separation of ajmaline from other alkaloids with extremely similar structures and basicities. These alkaloids can be present in the drug with ajmaline, according to the provenance of the drug.

coefficient of variation of 1.34%. The values obtained demonstrate the good reproducibility of the detection and quantification system.

Different amounts of ajmaline were also analysed, calibration being effected with a sample of medium concentration. The theoretical amounts of ajmaline com-

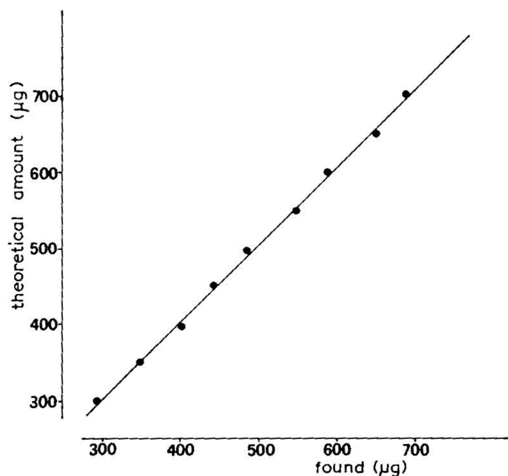


Fig. 3. Linearity of the chromatographic system in the range selected for analysis.

pared with those found experimentally are shown in Fig. 3. In the range selected, which is that normally valid for an analysis, a linear response is evident.

Recovery and reproducibility of the analytical method for the drug

A known amount of ajmaline (100 mg) was analysed five times following the methods of extraction and analysis described for the drug; there was no loss of the product during the preparation of the sample for analysis. The recoveries were: 98.7, 100.9, 99.8, 101.2 and 100.2 mg with an average value of 100.16 mg.

Ten analyses of the same batch of drug were carried out; the results obtained were 2.41, 2.55, 2.55, 2.52, 2.22, 2.41, 2.43, 2.36, 2.44, 2.45% (w/w) of ajmaline with a mean of 2.43% (w/w), a standard deviation of 0.098% (w/w) and a coefficient of variation of 4.05%.

CONCLUSIONS

This method has been used in our laboratories during the last 2 years and has been found suitable for the rapid, specific and precise determination of ajmaline in the bark of the root of *Rauvolfia vomitoria*. It seems that it could also be used for the examination of ajmaline in other drugs; it may also be used in the determination of the other alkaloids reported.

ACKNOWLEDGEMENTS

The author expresses his thanks to Mr. V. Cattani for technical assistance and to Mr. F. Boffi for the preparation of the diagrams.

REFERENCES

1 H. Schmitt and H. Schmitt, *Arch. Int. Pharmacodyn.*, 127 (1960) 163.
2 E. Volkner and P. Gaida, *Z. Inn. Med.*, 19 (1964) 123.
3 H. Kleinsorge, *Med. Klin.*, 54 (1959) 409.
4 H. Kleinsorge and A. Seifert, *Med. Klin.*, 60 (1965) 825.
5 G. Forster and M. Holzmann, *Schweiz. Med. Wochenschr.*, 97 (1967) 185.
6 C. Bianchi and E. Beccari, *Minerva Med.*, 57 (1966) 1273.
7 M. S. Habib and W. E. Court, *Can. J. Pharm. Sci.*, 8 (1973) 81.
8 W. E. Court and M. S. Habib, *J. Chromatogr.*, 80 (1973) 101.
9 Z. Jung and M. Jungova, *Cesk. Farm.*, 22 (1973) 195.

Journal of Chromatography, 176 (1979) 134–139
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,866

Note

Sensitive gas chromatographic method for the determination in blood and urine of SL 75212 [4-(2-cyclopropylmethoxyethyl)-1-phenoxy-3-isopropylaminopropan-2-ol], a new β_1 adrenoceptor blocking agent

G. BIANCHETTI, J. GANANSIA and P. L. MORSELLI
Synthelabo (L.E.R.S.), Department of Clinical Research, 58 rue de la Glacière, 75013 Paris (France)
(Received March 1st, 1979)

SL 75212 [4-(2-cyclopropylmethoxyethyl)-1-phenoxy-3-isopropylaminopropan-2-ol] is a new β-adrenoceptor blocking agent which has been shown, in animals and in preliminary clinical studies, to possess a very interesting β_1 selective profile[1,2]. The determination of the pharmacokinetic profile of a new therapeutic agent may be important for the better understanding of its mechanism of action and for more efficient therapeutic application. Further, the determination of drug concentrations in body fluids is important for defining possible therapeutic and/or toxic thresholds.

To evaluate the concentration of the drug in human fluids in order to study its clinical pharmacokinetic profile, we have developed a specific and sensitive gas–liquid chromatographic (GLC) assay for SL 75212.

EXPERIMENTAL

Standards and reagents

SL 75212 hydrochloride was synthesized by Dr. Manoury, Chemistry Department, Synthelabo (L.E.R.S.), and [^{14}C]SL 75212 was synthesized by C.E.A. (Saclay, Gif-sur-Yvette, France). Propranolol (I.C.I., Macclesfield, Great Britain) was used as an internal standard. Their structural formulae are shown in Fig. 1.

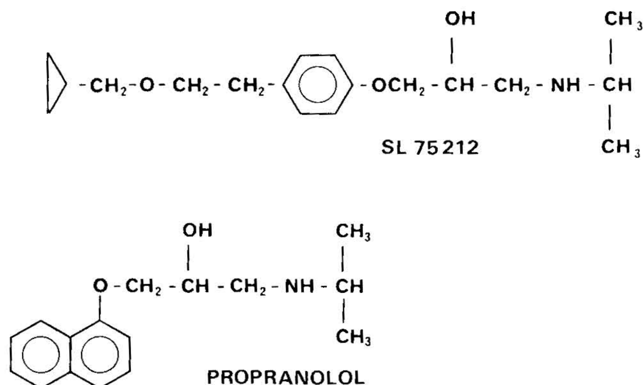


Fig. 1. Structural formulae of SL 75212 and the internal standard, propranolol.

The following reagents (all purchased from Merck, Darmstadt, G.F.R.) were used: diethyl ether, ethyl acetate, sodium hydroxide, 37% hydrochloric acid, *n*-hexane and methanol; heptafluorobutyric anhydride (HFBA) (puriss p.a.) was obtained from Fluka (Buchs, Switzerland).

Gas–liquid chromatographic conditions

Analyses were performed under isothermal conditions on a Perkin-Elmer Model 3920B gas chromatograph equipped with a ^{63}Ni linear electron-capture detector operating at −55 V (pulse current) with a 250 nsec width.

The glass column (2 m × 3 mm I.D.) was packed with Chromosorb W AW DMCS (80–100 mesh) coated with 3% OV-17, (Applied Science Labs, State College, Pa., U.S.A.) and conditioned for 1 h at 270° (40 ml/min argon–methane carrier gas flow), 4 h at 320° (no gas flow) and 24 h at 280° (50 ml/min carrier gas flow). The column temperature was 210°, injection port temperature 280°, interface and detector temperature 300° and carrier gas flow-rate 50 ml/min.

Mass spectrometric conditions

An LKB-2091B gas chromatograph–mass spectrometer with a PDP 11 data system was used. Spectra were obtained under the following conditions in the electron-impact mode: electron energy 70 eV, trap current 100 μA, accelerating voltage 3.5 kV, ion source temperature 250° and molecular separator temperature 250°. For GC, the same column was used as described under *Gas–liquid chromatographic conditions*, except that the helium flow was reduced to 20 ml/min.

Calibration graph and quantitation

Standard solutions of SL 75212 (1 μg/ml) and the internal standard propranolol (1 μg/ml) were prepared in methanol. These solutions were stable for at least 2 months.

Internal standards were prepared by adding 5, 10, 20, 40 and 80 ng of SL 75212 to 1 ml of blank blood and the samples were extracted according to the method described below. The extracts were then derivatized by heating with HFBA at 50° for 15 min, evaporated to dryness under a gentle stream of nitrogen and, when dried, the nitrogen flow was increased for a further 5 min in order to remove compounds derived from the reaction mixture which would appear as interfering peaks on the chromatogram. After dissolution in 200 μl of *n*-hexane, 1 μl of the standard solution of the derivative was injected. The ratio of the peak areas of SL 75212 to internal standard was used to calculate a response factor which was then used by a Perkin-Elmer PEP-2 data system to evaluate the amount of drug in the unknown samples.

Extraction procedure for blood

To 0.5–2 ml of human blood, in 10-ml glass-stoppered test-tubes, were added 20 ng of propranolol (20 μl of the methanolic stock solution) as internal standard, 100 μl of 2 *N* sodium hydroxide solution and distilled water to give a final volume of 3 ml. To this were added 6 ml of freshly distilled diethyl ether. The tubes were gently mixed on a rotating mixer for 15 min and then centrifuged at 4° for 5 min at 800 *g*. The ether phase was transfered to another series of test-tubes containing

2.5 ml of 0.2 *N* hydrochloric acid, mixed for 15 sec on a vortex mixer and centrifuged for 2 min at 800 *g*. The resulting upper ether phase was discarded. A further 5 ml of diethyl ether were added to the aqueous phase and the agitation and the centrifugation repeated. After discarding the ether phase, 300 μl of 2 *N* sodium hydroxide solution were added to the aqueous phase together with 5 ml of diethyl ether and the tubes were mixed on a vortex mixer and centriguged as above. The ether phase was transferred to another series of tubes and evaporated under nitrogen in a water-bath at 40°.

A 200-μl volume of HFBA (1:10 solution in ethyl acetate) was then added to the dry residue and the samples were derivatized as described above.

An internal calibration graph (five points) with various amounts (10–80 ng/ml) of SL 75212 added to the blood was always carried through the procedure with the unknown samples.

Extraction procedure for urine

The same procedure of extraction and derivatization was carried out for measuring SL 75212 in urine. The only difference consisted in the volume of sample, which was always less than 1 ml (0.1–0.5 ml).

RESULTS AND DISCUSSION

The GLC trace obtained from a blood sample to which a known amount of SL 75212 had been added is shown in Fig. 2, together with those obtained from a blood blank and from a volunteer receiving SL 75212 by the oral route. The peaks of SL 75212 and propranolol are well resolved and no interfering peaks from endogenous substances are present.

The GLC–MS analysis confirmed the identity of the gas chromatographic peaks. GLC peaks due to the reaction product of SL 75212 and HFBA showed a molecular ion at *m/e* 699 in the mass spectrum, corresponding to the formation of the diheptafluorobutyrate (Fig. 3).

Another characteristic peak in the spectrum was that at *m/e* 508, corresponding to the loss of a cyclopropylmethoxyethyl-4-phenoxy group. This ion loses a fragment of 43 a.m.u. [H_3C–$\dot{C}H$–CH_3] to give the base peak at *m/e* 465, which in turn loses a fragment of 213 a.m.u. ($\cdot OCOCF_2CF_2CF_3$) to give an ion at *m/e* 252. An ion at *m/e* 55, corresponding to the methylisopropyl group, is also present.

The GLC characteristics of SL 75212 and propranolol are reported in Table I (retention time, height equivalent to a theoretical plate, resolution and symmetry factor).

A standard calibration graph obtained after extraction of SL 75212 from blood is shown in Fig. 4, and the coefficients of variation at different concentrations are reported in Table II.

The absolute recovery of SL 75212, calculated by using ^{14}C-labelled SL 75212, was 86–90%; one extraction was considered to be adequate.

The absolute sensitivity of the electron-capture detector was about 20 pg for SL 75212 and 5 pg for propranolol; this means that it is possible to detect 1 ng/ml of SL 75212 by increasing to 2–4 μl the volume of the final solution injected on to the gas chromatographic column.

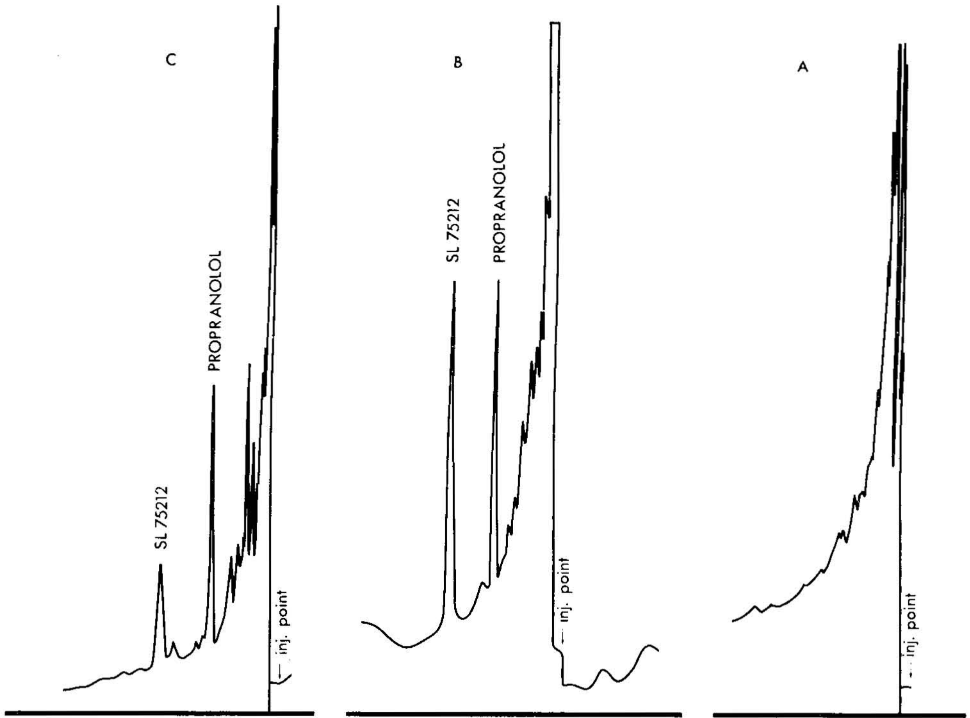


Fig. 2. Gas chromatograms of plasma extracts. (A) Plasma free from drug; (B) plasma to which known amounts of propranolol (20 ng) and SL 75212 (50 ng) have been added; (C) plasma of a volunteer who took 10 mg of SL 75212 orally.

The HFBA derivative of SL 75212 is very stable and the ratio between the amounts of SL 75212 and propranolol did not change during 1 week when the solution was kept at room temperature.

No interfering peaks due to endogenous substances or other cardiovascular drugs were noted. The analysis of blood samples containing guanethidine, clonidine, chlorthalidone, furosemide, lidocaine and quinidine showed that none of these substances interfered with the assay. Other β-blocking agents, such as alprenolol, oxprenolol and metoprolol, gave retention times lower than those of SL 75212 and propranolol.

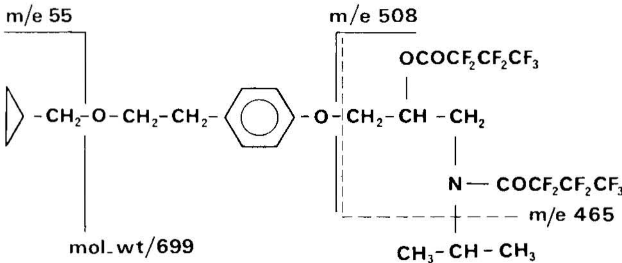


Fig. 3. Fragmentation pattern of the HFBA-derivative of SL 75212.

TABLE I

GAS CHROMATOGRAPHIC CHARACTERISTICS OF SL 75212 AND PROPRANOLOL (INTERNAL STANDARD)

Parameter	*SL 75212*	*Propranolol*
Retention time (min)	7.30	4
Height equivalent to a theoretical plate (mm)	1.37	1.57
Resolution	5.6	8.5
Symmetry factor of peak	0.95	0.96

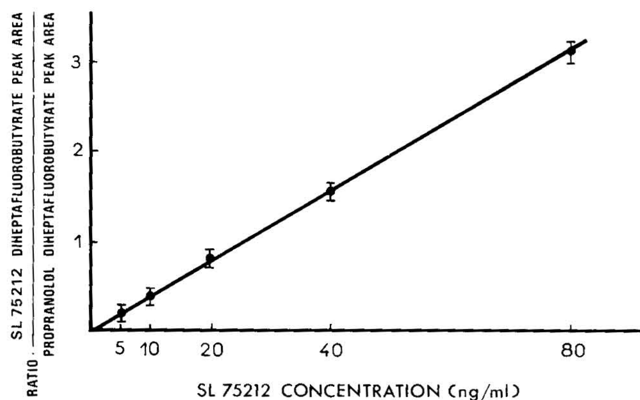


Fig. 4. Standard calibration graph for SL 75212.

TABLE II

ACCURACY OF DETERMINATION OF SL 75212 IN HUMAN BLOOD

Concentration of SL 75212 (ng/ml)	*Mean concentration found ± S.D. (ng/ml)*	*Coefficient of variation (%)*	*Number of determinations*
10	10.8 ± 0.8	7.4	10
20	18.6 ± 0.9	4.8	10
40	40.6 ± 1.2	2.9	10
60	62.4 ± 1.1	1.7	10

TABLE III

SL 75212 BLOOD CONCENTRATIONS IN VOLUNTEERS AT PEAK TIMES AND 24 h AFTER ADMINISTRATION OF A SINGLE DOSE OF THE DRUG ORALLY

Dose (mg)	*Concentration (ng/ml)*	
	2 h	*24 h*
5	10.5	3
5	10.0	5.2
10	16.5	10.5
10	27.0	6.0
20	34.0	13.0
20	49.5	16.0
40	51.5	18.0
40	69.5	19.0

The method described has been applied to the determination of SL 75212 in the blood of volunteers receiving a single dose of the drug by the oral route[3]. The concentration of SL 75212 in blood could be detected even when the smallest doses were given (5 mg). In Table III are reported some values of the concentration of SL 75212 in blood at peak concentration times and 24 h after administration of different doses of the drug. It can be seen that the amount of SL 75212 in blood was directly related to the dose administered.

CONCLUSION

A sensitive and specific method for the determination of SL 75212 has been developed, which provides a relatively simple procedure for all the future pharmacokinetic studies on SL 75212 and also allows the dtermination of blood levels of the drug on a routine basis. The time necessary to prepare 20 samples for GLC injection does not exceed 2 h.

ACKNOWLEDGEMENTS

The authors are indebted to Mr. V. Rovei for the mass spectrometric analyses and their interpretation and to Dr. L. G. Dring for helpful discussions regarding the manuscript.

REFERENCES

1 J. F. Boudot, I. Cavero, S. Fenard, F. Lefevre-Borg, P. Monoury and A. G. Roach, *British Pharmacology Society Meeting, Dublin, July 18, 1979.*
2 B. L. Pentecost, P. J. Cadigan, D. London, G. Bianchetti, J. Kilborn and P. L. Morselli, *British Pharmacology Society Meeting, Dublin, July 18, 1979.*
3 J. F. Giudicelli, C. Thuilliez, C. Richer, G. Bianchetti, R. Gomeni and P. L. -Morselli, *British Pharmacology Society Meeting, Dublin, July 18, 1979.*

Journal of Chromatography, 176 (1979) 140–144
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,831

Note

Analyse par chromatographie liquide haute pression de la décaméthrine

C. MEINARD, J. C. SUGLIA et P. BRUNEAU

Division Scientifique, Roussel-Uclaf-Procida, Département de Biologie Appliquée St. Marcel 13011 Marseille (France)

(Reçu le 14 novembre 1978; manuscrit modifié reçu le 27 février 1979)

La décaméthrine (Décis®, NRDC 161) (*S*)-*α*-cyano-3-phénoxybenzyl-(1*R*, 3*R*)-3-(2,2-dibromovinyl)-(2,2-diméthylcyclopropane carboxylate) est un pyréthrinoïde découvert par Elliott *et al.*[1–3] dont la synthèse stéréospécifique a été maîtrisée par Roussel-Uclaf.

En effet, le produit comporte trois carbones asymétriques soit 2^3 (8) stéréoisomères et 2^2 (4) couples d'énantiomères diastéréoisomères.

Parmi ces 8 stéréoisomères, celui qui possède la meilleure activité insecticide est la décaméthrine. Certains mélanges d'isomères ont été décrits par Elliott *et al.*[1–3]: le NRDC 156 et le NRDC 158. Le NRDC 156 est le mélange des diastéréoisomères *cis R* et *S* quant au carbone portant la fonction nitrile (*cf.* Fig. 1); le NRDC 158 correspond au même mélange en série *trans*.

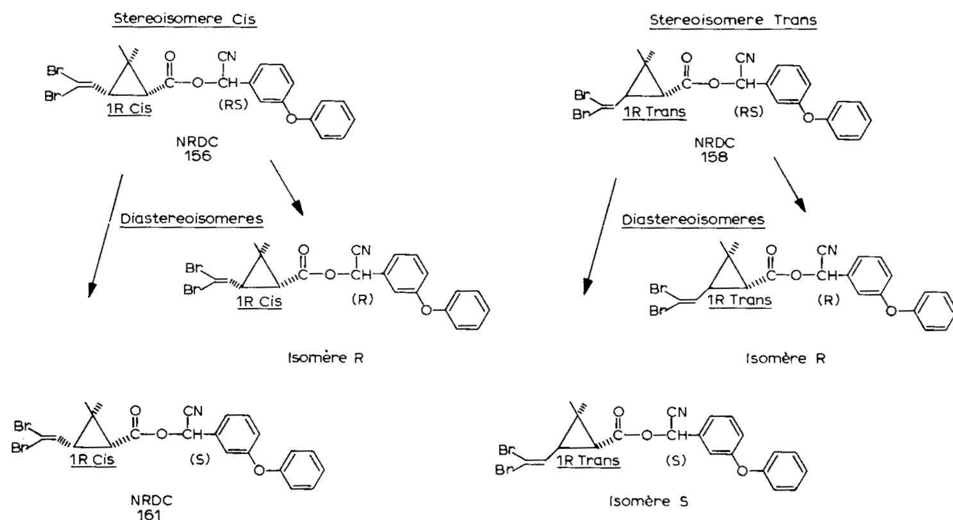


Fig. 1. Mélanges d'isomères.

Il était donc nécessaire de trouver une méthode d'analyse qui permette l'identification spécifique de la décaméthrine en présence des trois autres diastéréoisomères sans risque d'interférence.

La chromatographie liquide haute pression (HPLC) a pu nous permettre cette identification ainsi que le dosage spécifique de la décaméthrine. En effet, elle nous a permis de séparer les stéréoisomères *cis* et *trans* ainsi que leur diastéréoisomère respectif *R* et *S* (*cf.* Fig. 1) ce que nous n'avions pu obtenir par chromatographie phase gazeuse (GLC). Il était utile de posséder une technique permettant de pouvoir doser uniquement un des stéréoisomères ((*S*)-décaméthrine) produit biologiquement plus actif que le stéréoisomère *R*. Afin de suivre l'évolution du produit et connaître ses éventuelles transformations en son stéréoisomère (*R*).

La technique par HPLC a été utilisée par Kikta et Shierling[4] et Lam et Grushka[5] pour séparer les stéréoisomères *cis* et *trans* de la perméthrine ainsi que leurs métabolites.

CONDITIONS EXPÉRIMENTALES

Appareillage et réactifs utilisés

Chromatographie Varian 8500 permettant d'atteindre des pressions de l'ordre de 8500 p.s.i. soit 600 bar et de travailler à débit constant. Détecteur multilongueur d'onde Variscan permettant de se placer au maximum d'absorption du produit étudié dans notre cas $\lambda = 224$ nm. La colonne une Micropak SI 10, longueur: 50 cm (diamètre intèrieur 1/8 pouce, diamètre extérieur 1/4 pouce). Les solvants de qualité spectro UV. Les produits étant le NRDC 161 (décaméthrine pure), NRDC 158 et étalon: le phtalate de dioctyle.

Conditions opératoires

Mélange éluants: hexane (280 ml), pentane (113 ml), ether diéthylique (7 ml). Débit: 40 ml/h, sensibilité du détecteur: 0.5, vitesse de déroulement du papier: 50 cm/h.

ANALYSE QUALITATIVE ET QUANTITATIVE

Analyse qualitative — identification des stéréoisomères cis et trans ainsi que leurs diastéréoisomères

En utilisant les conditions ci-dessus et en injectant soit le NRDC 158, soit le NRDC 156 ou le mélange de ces deux isomères géométriques, nous observons sur le chromatogramme I (Fig. 2) que l'isomère *cis* est élué avant l'isomère *trans* et que nous séparons les deux diastéréiosomères *R* et *S* bien distinctement, sans aucune superposition de signaux, ce que était important pour la suite du dosage. L'analyse complète des quatre isomères dure 40 min.

Analyse quantitative — Dosage de la décaméthrine

Pour réaliser cette analyse quantitative, nous avons fait appel à un étalon interne qui soit valable pour ces quatre isomères: notre choix s'est porté sur le phtalate de dioctyle.

Courbe étalon de la décaméthrine. Préparations des solutions:

(1) Solution de phtalate de dioctyle d'environ 25 g/l: 2.5108 g de phtalate de dioctyle q.s.p. 100 ml avec hexane–éther diéthylique (70:30).

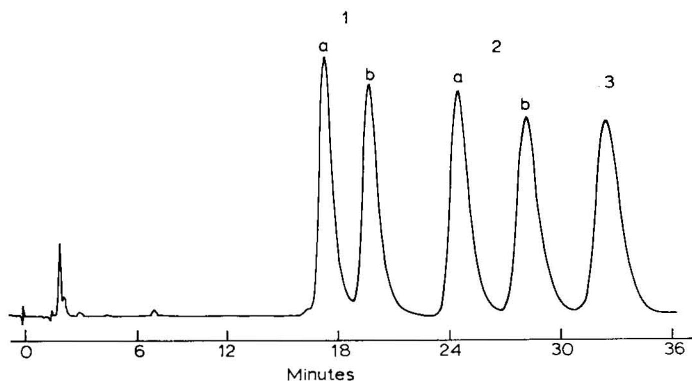


Fig. 2. Séparation des isomères *cis* NRDC 156 et des isomères *trans* NRDC 158 et de leur diastéréoisomères *R* et *S* respectifs. Conditions: échantillon injecté, 9.6 μg d'échantillon dans 3 μl de la phase mobile; colonne Micropak SI 10, 50 cm × 3.18 mm (D.I.); phase mobile, hexane–pentane–éther diéthylique (280:113:7); pression, 25 bar; débit, 40 ml/h; détecteur U.V., 224 nm; vitesse de déroulement du papier, 50 cm/h; étalon interne, phtalate de dioctyle. 1 = Isomère *cis*: NRDC 156; 2 = isomère *trans*: NRDC 158; 3 = étalon interne; a = diastéréoisomère *R*; b = diastéréoisomère *S*.

(2) Solutions de la décaméthrine (q.s.p. 25 ml avec hexane–éther diéthylique (70:30):

No.	*Poids de décaméthrine à 100% (mg) dans 5 ml de solution (1)*
1	10.93
2	15.62
3	20.14
4	24.44
5	30.44
6	39.50
7	48.62
8	58.92
9	68.38
10	76.90

Calcul. 3 μl des solutions préparées au paragraphe *Analyse quantitative — Dosage de la décaméthrine* sont injectés dans l'appareil. Les conditions opératoires sont celles définies dans le paragraphe Conditions expérimentales. L'intégration est obtenue par le produit de la hauteur du pic par la largeur à mi-hauteur. Après intégration des pics obtenus, on trace le graphe:

$$\frac{\text{Poids de décaméthrine}}{\text{Poids de phtalate de dioctyle}} = \mathrm{f}\,\frac{\text{(surface du pic de la décaméthrine)}}{\text{(surface du pic du phtalate de dioctyle)}}$$

$$Y = \mathrm{f}(X)$$

C'est une droite passant par l'origine (Fig. 3).

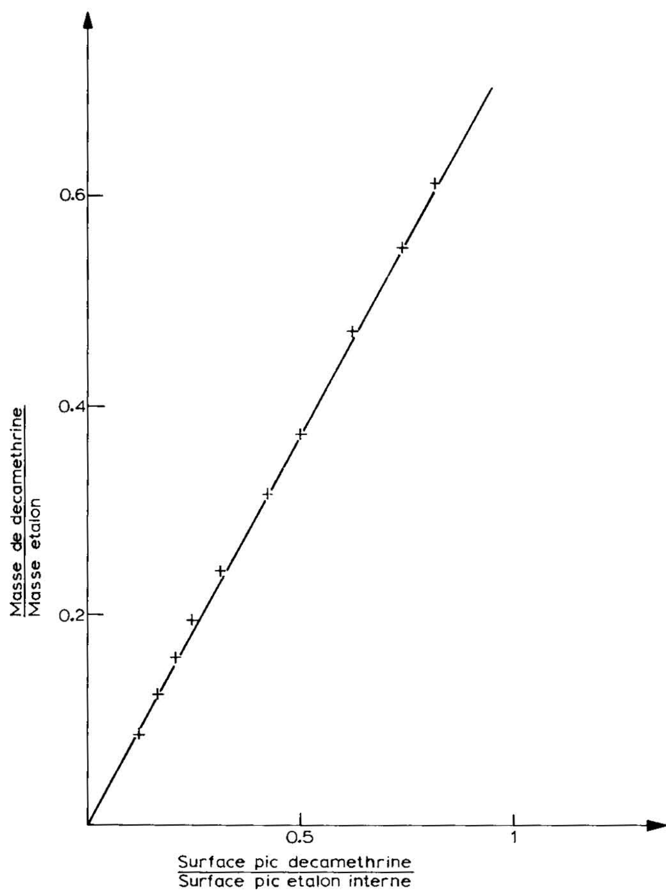


Fig. 3. Courbe d'étalonnage de la décaméthrine par la méthode de l'étalon interne. Rapport des masses décaméthrine–étalon interne en fonction des aires décaméthrine–étalon interne.

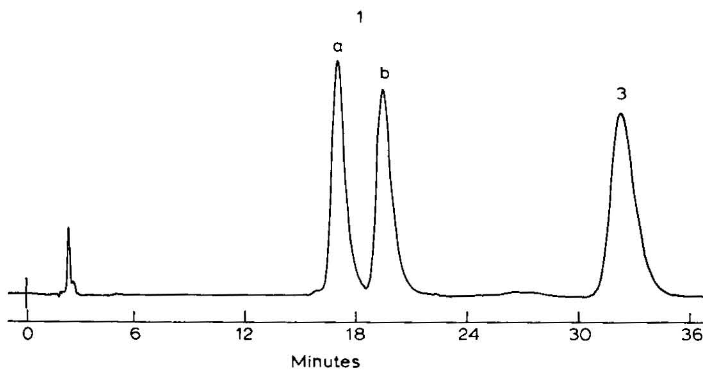


Fig. 4. Dosage de la décaméthrine dans le mélange NRDC 156. 1 = Isomère *cis*: NRDC 156; 3 = étalon interne; a = diastéréoisomère *R*; b = diastéréoisomère *S* (NRDC 161)-décaméthrine. Même conditions opératoires que celles décrites pour la Fig. 2.

APPLICATION—DOSAGE DE LA DÉCAMÉTHRINE DANS LE MÉLANGE NRDC 156

Peser environ 80 mg de mélange (79.99 mg) dans une fioles de 25 ml; ajouter 5 ml de la solution de phtalate de dioctyle préparée au paragraphe *Analyse quantitative — Dosage de la décaméthrine*. Compléter à 25 ml avec hexane–éther (70:30). Injecter 3 μl de la solution obtenue (Fig. 4).

Après intégration, on obtient $X = 0.421$; la courbe étalon donne $Y = 0.315$, d'où le titre:

$$T = \frac{0.315 \cdot 125.54 \cdot 100}{79.99} = 49.4\,\%\ (\text{w/w})$$

D'une manière générale:

$$T = \frac{Y \cdot E \cdot 100}{P}$$

où E = poids de phtalate de dioctyle et P = poids de mélange (dans les 25 ml de solution).

CONCLUSION

Cette méthode par HPLC nous permet de doser d'une manière spécifique la décaméthrine en présence de mélange d'isomères (isomère R contenu dans les stéréoisomères *cis* ainsi que les deux isomères R et S du stéréoisomère *trans*), sans aucune intérférence. Ce dosage spécifique de l'un des diastéréoisomères est un des avantages de cette méthode qui semble plus adaptée que la GLC pour ces molécules complexes.

BIBLIOGRAPHIE

1 M. Elliott, A. W. Farnham, N. T. Janes, P. H. Needham et D. A. Pulman, *A.C.S. Symp. Ser.* No. 2 (1974) 80.
2 M. Elliott, A. W. Farnham, N. T. Janes, P. H. Needham et D. A. Pulman, *Nature (London)*, 248 (1974) 710.
3 M. Elliott, A. W. Farnham, N. T. Janes, P. H. Needham et D. A. Pulman, *Pestic. Sci.*, 6 (1975) 537.
4 E. J. Kikta, Jr. et J. P. Shierling, *J. Chromatogr.*, 150 (1978) 229.
5 S. Lam et E. Grushka, *J. Chromatogr.*, 154 (1978) 318.

Journal of Chromatography, 176 (1979) 145–147
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,890

Note

Identification and separation of closely related sulfa drugs by thin-layer chromatography on cadmium acetate-impregnated silica gel plates

S. P. SRIVASTAVA, V. K. DUA, R. N. MEHROTRA and R. C. SAXENA
Chemical Laboratories, University of Roorkee, Roorkee (India)
(First received September 6th, 1978; revised manuscript received March 27th, 1979)

Recently the impregnation of thin-layer plates with different substances has been tried in order to improve the separation of various classes of substances. Yasuda[1–4] reported the use of different metal salts for the separation of aromatic amines, while Srivastava and Dua, in this laboratory, have developed suitable impregnants for the separation of phenols[5], aromatic amines[6], aliphatic amines[7], diols[8] and amino acids[9] by thin-layer chromatography (TLC). The loading of papers with strong acid exchange resin has been employed by Pietrzyk and Chan-Santos[10] for the separation of some sulfa drugs. However, no work seems to have been done, on the TLC separation of sulfa drugs on impregnated plates. The present paper reports our studies on the use of impregnated silica gel plates for the separation and identification of ten sulfa drugs.

EXPERIMENTAL

The plates (0.5 mm thick) were coated with a mixture of silica gel (50 g) and 1% metal salt solution (100 ml), and then activated at 60 $\pm$ 1° for 24 h. Solutions of sulfa drugs (0.05% w/v) in ethanol were used for spotting. After development the chromatoplates were sprayed with DAB (50 mg of *p*-dimethylaminobenzaldehyde dissolved in 1 ml concentrated sulphuric acid and made up to 100 ml with 95% ethanol)[11]. Yellow spots appeared on a white background.

The various impregnants tried were cadmium sulphate, cadmium acetate, zinc sulphate, zinc acetate, manganese sulphate and manganese acetate. In all cases, a 1% solution of the impregnant gave the best separation. On cadmium acetate all ten sulfa drugs were separated when present in the mixture. The most suiteble solvent system was found to be toluene–benzene–chloroform–ethyl methyl ketone–methanol (100:20:25:25:25). Some representative separations of sulfa drugs on metal salt-impregnated plates are given in Table I.

RESULTS

Comparison of the data in Table I shows that by using a metallic salt as impregnant the separation of different sulfa drugs is quite satisfactory and that

TABLE I

REPRESENTATIVE SEPARATIONS OF SULFA DRUGS ON TLC PLATES IMPREGNATED WITH METAL SALTS

The $R_F \cdot 100$ values reported are means from two or more identical runs. Rate of development: 12 cm in 30 min.

Sulfa drug	*Impregnant*						*Detection limit (ng)*
	Cadmium sulphate	*Cadmium acetate*	*Zinc sulphate*	*Zinc acetate*	*Manganese sulphate*	*Manganese acetate*	
Sulfaphenazole	—	68	70	60	68	72	23
Sulfamethizole	22	4	24	3	—	—	13
Sulfasomidine	27	10	43	36	45	42	37
Sulfadimidine	55	38	56	52	—	65	22
Sulfaguanidine	33	23	30	18	23	22	35
Sulfadiazine	46	18	—	—	54	51	10
Sulfathiazole	42	14	—	—	—	37	16
Sulphanilamide	—	45	—	—	—	—	35
Sulfamerazine	50	29	—	42	60	56	37
Sulfapyridine	—	34	52	—	—	—	44

there is no tailing. On cadmium acetate-impregnated plates the size of the spots is minimal and all the sulfa drugs are separated. The R_F values of the sulfa drugs do not change when present in a mixture. Manganese acetate as an impregnant results in equally good separations but only seven sulfa drugs can be separated on this adsorbent system, while only five or six sulfa drugs can be separated using cadmium sulphate, zind sulphate, zinc acetate or manganese sulphate as impregnant.

The behaviour of the sulfa drugs on impregnated plates depends on the following two factors:

(i) The formation of a metal–sulfa drug complex involving the metal ion and the amino group of the sulfa drug, as was suggested by Yasuda[1] in the case of aromatic amines. This is supported by the observation of Narang and Gupta[12] who reported the formation of a complex between copper(II) and a sulfa drug.

(ii) The formation of hydrogen bonding due to the hydrogen atoms of the NH_2 group and oxygen atoms of the acetate or sulphate anion, as reported by Srivastava and Dua[9] in the case of TLC separation of amino acids on calcium oxalate-impregnated plates.

Thus we see that the movement of sulfa drugs is governed by the nature of both the metal ion and the anion. Besides the above two factors, steric effects and solvation factors should also influence the R_F values. Further work in this direction is in progress.

REFERENCES

1 K. Yasuda, *J. Chromatogr.*, 60 (1971) 144.
2 K. Yasuda, *J. Chromatogr.*, 72 (1972) 413.
3 K. Yasuda, *J. Chromatogr.*, 74 (1972) 142.
4 K. Yasuda, *J. Chromatogr.*, 87 (1973) 565.
5 S. P. Srivastava and V. K. Dua, *Z. Anal. Chem.*, 275 (1975) 29.
6 S. P. Srivastava and V. K. Dua, *Z. Anal. Chem.*, 276 (1975) 382.

7 S. P. Srivastava and V. K. Dua, *Z. Anal. Chem.*, 279 (1976) 367.
8 S. P. Srivastava and V. K. Dua, *Z. Anal. Chem.*, 286 (1977) 247.
9 S. P. Srivastava and V. K. Dua, *Indian. J. Chem.*, 15A (1977) 761.
10 D. J. Pietrzyk and E. Chan-Santos, *J. Chromatogr.*, 87 (1973) 543.
11 S.-C. Lee, *J. Chromatogr.*, 93 (1974) 480.
12 K. K. Narang and J. K. Gupta, *Ind. J. Chem.*, 13 (1975) 705.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* 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 reviews, see page 2 of cover under Submission of Papers.

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.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. 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 authors' 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. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

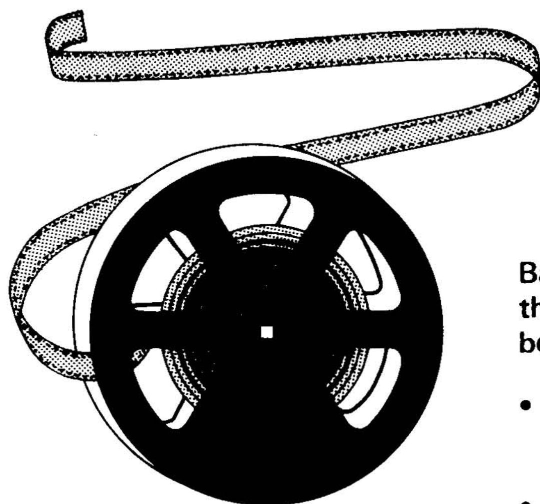
Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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.

This journal now available on MICROFILM



Back volumes up to the end of the 1978 subscription year may be obtained on microfilm

- **Annual Updates** – in Spring of each year, volumes published the previous year will become available on microfilm
- **Complete Sets** – a 10% discount is included in the price of complete sets which comprise all available volumes on microfilm of one title
- **Single Volumes** – Individual volumes may be purchased of any title
- **Continuation Orders** – Purchasers who enter a continuation order will automatically receive a proforma invoice for the annual updates

Microfilm Specifications

- 16 mm or 35 mm: positive or negative film supplied in plastic labelled storage boxes: cartridge or cassette
- Polyester base silver halide film
- Reduction ratio 24 X
- Guaranteed standards – NBS resolution charts on each 100 ft reel
- 2A cine mode double page spread across the film

Orders and information requests concerning back volumes on microfilm should be addressed exclusively to:

ELSEVIER SEQUOIA S.A.
P.O. Box 851
CH-1001 **Lausanne 1**
Switzerland

297 Sa

18.ศ.ค. 2522