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Albert Zlatkis Honour Volume

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INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

SYMPOSIUM VOLUMES

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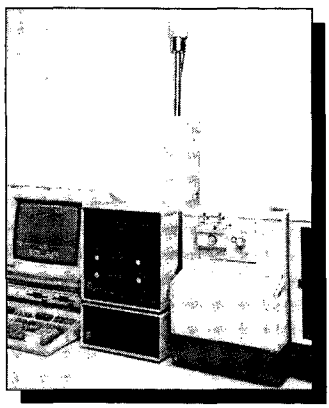

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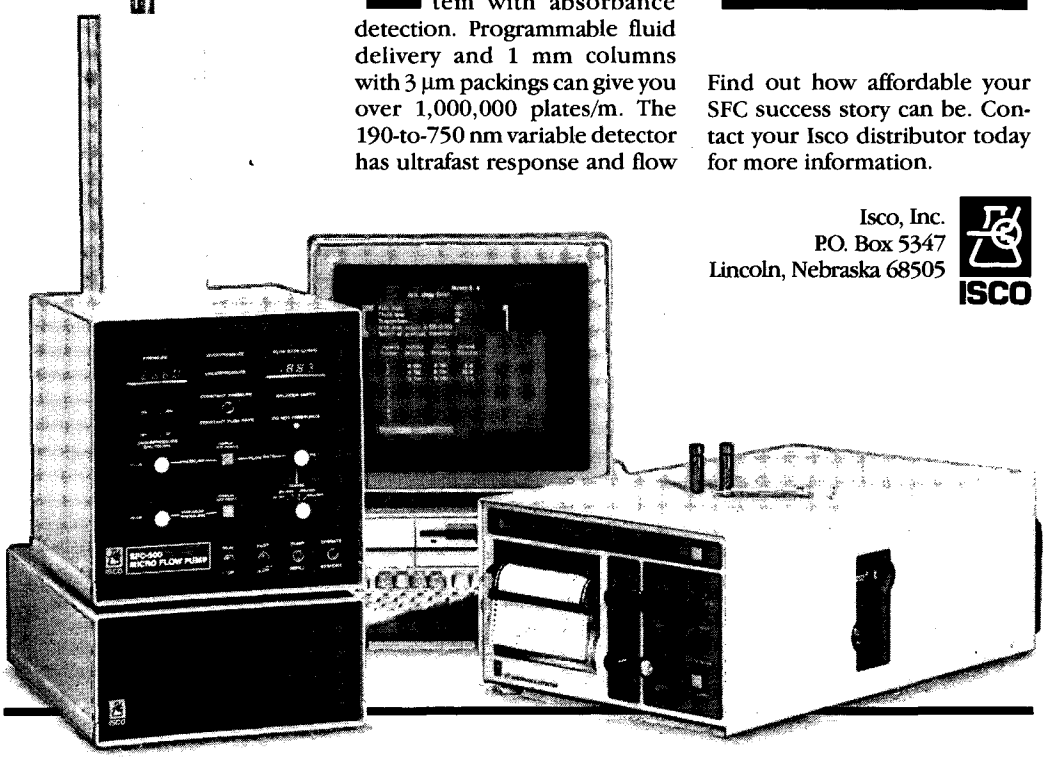

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SYMPOSIUM VOLUME

ADVANCES IN CHROMATOGRAPHY 1988

Proceedings of the **Twenty-fifth Anniversary** International Symposium held in Minneapolis, MN, August 29–September 1, 1988

Guest Editors

A. ZLATKIS
(Houston, TX, U.S.A.)

L. S. ETTRE
(Norwalk, CT, U.S.A.)

B. S. MIDDLEDITCH
(Houston, TX, U.S.A.)

This volume is dedicated to

ALBERT ZLATKIS

on the occasion of his 65th birthday

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FOREWORD

One of the books that I read during my first years as a graduate student was *Little Science, Big Science*, by Derek J. de Solla Price. He devoted an entire chapter to the concept of the Invisible College, a *de facto* group of colleagues who could avoid the effects of the information explosion by exchanging information directly rather than having to glean it from the burgeoning literature. In his "Survey of Chemical Publications" compiled for The Chemical Society, R. S. Cahn decried this approach on the grounds that "an élite will be well-informed and the remainder ill-informed". What hope was there for a new graduate student who wished to avoid drowning in the flood of literature that he was inundated with?

Fortunately for me and others who were new to the field, the science of chromatography had already spawned a Visible College. Under the stewardship of Professor Zlatkis it held regular symposia that were open to all. There were no qualifications for admission and no dues to pay. One did not even have to attend the meetings since —in those early days— the proceedings were published in time for distribution at the symposia.

A key to the success of this Visible College was that only a limited number of papers were included in the program, and that they were generally presented by the senior authors in person. There were rarely any parallel sessions or stumbling presentations by students. The leaders in our field saved their very best data each year for this prestigious symposium. The proceedings of the symposia —always published concurrently in refereed journals— provide a complete chronicle of the major advances in chromatography over the past quarter century.

The Silver Jubilee Symposium was no exception to this tradition. Some of the original faculty of this Visible College are no longer with us, but their successors have continued to demonstrate that the science of chromatography can be adapted to the solution of even the most challenging of problems.

Houston, TX (U.S.A.) BRIAN S. MIDDLEDITCH

All *International Symposia on Advances in Chromatography* (1963–1988) are listed in Table I (p. 2 in this volume) of the article by L. S. Ettre on the history of this series of symposia. The complete list of recipients of the M. S. Tswett Chromatography Medal (1974–1988) is given in Table IV (pp. 18/19 in this volume) of the same article.

AN INTRODUCTION AND A CELEBRATION

This volume serves a triple purpose. It represents the proceedings of the *Twenty-Fifth International Symposium on Advances in Chromatography* held August 29–September 1, 1988, in Minneapolis, Minnesota, *and* celebrates the Silver Jubilee of this symposium series. But it *also* celebrates the sixty-fifth birthday of Albert Zlatkis, Professor at the University of Houston, the founder of these symposia and the driving force behind each and every symposium since 1963. The speakers at the Minneapolis Symposium were aware of this forthcoming anniversary and contributed their papers with all three goals in mind.

Albert Zlatkis was born on March 27, 1924, in the Polish–Ukrainian–Russian plains, immigrating to Canada with his parents at the age of three, as a late-coming member of the "huddled masses yearning to breathe free"[1]. He spent the next 21 years in Toronto, where he studied chemical engineering and received his B.A.Sc. and M.A.Sc. degrees. Accepting a fellowship to Wayne State University, in Detroit, Michigan, U.S.A., he continued his studies, which led to his Ph.D. degree in 1952. He then went to Shell Oil Company, in Houston, Texas. It is well known that the worldwide Shell laboratories pioneered in the adaptation and further development of gas–liquid chromatography, immediately after the publication by James and Martin[2], and the Houston laboratory was part of these activities. In this way Al became involved in gas chromatography as early as 1954, a field he remained faithful to for his whole professional life.

At that time the University of Houston was a small school, with most of the teaching being done on a part-time basis by chemists and engineers associated with Houston industrial companies. Al was one of them. He liked teaching so much that when, in August 1955, he was offered a full-time assistant professorship at the University, he accepted it; this was one of the most important decisions in his life. He has been associated with this school ever since. From Shell he took with him his newly acquired knowledge in gas chromatography and immediately utilized it in trying to solve separation problems which, until then, seemed to be insolvable, such as the separation of enantiomers and close-boiling isomers. He also participated in the early gas chromatography symposia, building up contact with the leaders in the field, both in the U.S.A. and in Europe.

On the occasion of his sixtieth birthday, Dr. Zlatkis' professional career and achievements were discussed in detail in an Editorial[3], and I do not want to repeat them here. In the past five years his activities have mainly centered on the possibilities of ultra-trace analyses by gas chromatography. He has also collected two important awards to add to those already listed in my Editorial: he was named by the Texas Academy of Science as the Distinguished Texas Scientist for 1985; and, in 1988, he received the Southwest Regional Award of the American Chemical Society.

Albert Zlatkis is one of the best-known American chromatographers and thus, he really does not have to be introduced. How shall I describe him? Probably his most important characteristics are an openness and friendliness toward everybody, regardless of origin, social level, or knowledge; a faith in the goodwill of the members of the human race; a sixth sense in realizing what is important; and an immense energy and activity to carry out any task he considers worthwhile. He is a devoted family man who is lonely if only for one day he has to miss his wife or children. He believes in the

American dream that people can succeed if they are given the chance to do so. Therefore, he will help everybody who asks him; in fact, he will try to help even when he is not asked! But for Al, "helping" means to place somebody on a track: from then on it is up to the individual to achieve.

Today, the word "friend" is often misused, referring to everybody one knows, even if only casually. For Al, this word still retains its original meaning, referring to a person one is attached to by esteem, respect and affection, a person one can trust. Probably, because he values its true meaning, Al has many friends; also, there are many people who cherish the opportunity to call him their friend.

I will never forget my first meeting with Al. After immigrating to the U.S.A., I joined Perkin-Elmer at the beginning of October 1958. A few weeks later, suddenly a smiling person, as yet unknown to me, burst into our laboratory to great my colleagues with whom he just published a paper on the separation of the three xylene isomers by using benzyldiphenyl as the liquid phase[4]. I still had some of the reserved, more-or-less formal European manners and was astonished by his ability to dominate a place within seconds, in a completely informal way. I visited him in Houston about 10 months later, on the occasion of a meeting of the Gulf Coast Spectroscopic Group, when I also had the opportunity to be introduced to his wife, Esther. We have been in close contact ever since, a friendship I treasure very much.

It is said that at 65, people start to slow down. Frankly, I personally disagree with this philosophy, and I know that Al does too. Thus, we can surely expect many surprises from him in the years to come: what they will be, even I do not know. However, I am sure that they will be just as interesting and exciting as all the other endeavours in which Al has been involved, and I am certainly looking forward to participating in them.

On the occasion of his sixty-fifth birthday, I would like to convey to Al the best wishes of all those chromatographers who, in the past 25 years, participated in the symposia organized by him. We wish him many more productive years and a continuing enjoyment in our joint hobby: chromatography. And last but not least, I would like to thank Al personally for all the opportunities he gave me in our many joint projects over the past 30 years. I believe that what we did was useful and contributed to the evolution of the field in which we were active. It was a most rewarding and exciting experience —and, what is probably the most important: it was great fun.

Happy birthday Al!

LESLIE S. ETTRE

1 From *The New Colossus*, the poem by Emma Lazarus, which is engraved on the pedestal of the Statue of Liberty, in New York harbor.
2 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
3 L. S. Ettre, *Chromatographia*, 18 (1984) 233.
4 A. Zlatkis, L. O'Brien and P. R. Scholly, *Nature (London)*, 181 (1958) 1794.

THE M. S. TSWETT CHROMATOGRAPHY MEDAL

On the occasion of the *Silver Jubilee 25th International Symposium on Advances in Chromatography* held in Minneapolis, Minnesota, the M. S. Tswett Chromatography Medal was awarded to the following scientists in recognition of their contributions to the advances of chromatography.

PHYLLIS R. BROWN
FABRIZIO BRUNER
TSUNEO OKUYAMA

Their achievements in the advancement of science and the evolution of chromatographic techniques and methods are described in the following pages.

Minneapolis, MN (U.S.A.)
August 29, 1988

ALBERT ZLATKIS
Symposium Chairman

Phyllis R. Brown was born on March 16, 1924, in Providence, Rhode Island, U.S.A. She received her B.S. in chemistry at George Washington University, in Washington, D.C., U.S.A. After an educational hiatus of eighteen years, she returned to school and received her Ph.D. in chemistry in 1968 from Brown University, Providence, RI. She did postdoctoral work in the Pharmacology Section at Brown for three years and stayed on in that section as instructor and then as an assistant research professor. In 1973 she became an assistant professor in the Department of Chemistry at the University of Rhode Island, in Kingston, RI; in 1977 she was promoted to associate professor, and in 1980, to full professor. In 1983 Dr. Brown was a visiting professor at the Hebrew University in Jerusalem, Israel. Recently she was awarded a Fullbright Fellowship to return to Israel to continue her research there.

In 1985 Dr. Brown was awarded the scholarly Achievement Award for Excellence in Research at the University of Rhode Island, and also named Woman of the Year by the Business and Professional Women of South County, Rhode Island. In addition she was given in 1984 the Community Service Award by the Providence Chapter of the National Council of Jewish Women in 1984. The Dal Nogare Award in chromatography was presented to Dr. Brown in 1989.

Dr. Brown is the author and coauthor of over 100 scientific publications. She wrote the first book on biomedical and biochemical applications of high-performance liquid chromatography (HPLC) (Academic Press, 1973) and was the senior author of the first book devoted entirely to reversed-phase HPLC (Wiley, 1982). Both books were translated into Japanese. She edited a book on HPLC in nucleic acid research (Marcel Dekker, 1984) and edits, together with J. Calvin Giddings and Eli Grushka, the *Advances in Chromatography* series published by Marcel Dekker. She is on the editorial advisory boards of a number of scientific journals.

Dr. Brown has been a pioneer in the application of HPLC to biomedical research and has made outstanding contributions in the development of HPLC assays for biochemical research and the clinical laboratory. She is best known for her work in developing assays for nucleic acid constituents in biological samples. The HPLC methods she developed in 1970 for the separation of nucleotides in cell extracts are now the standard procedures used by biomedical researchers studying metabolism in normal subjects and patients with various diseases. She was a leader in systematizing methods for the identification of biologically important peaks and specifically, she developed the highly selective and sensitive enzyme peak shift technique for the identification of peaks in chromatograms of biological matrices. She was the first to use reversed-phase HPLC methods to determine concentrations of nucleotides and their bases in physiological fluids and established a range of normal values for these compounds in blood fluids.

Fabrizio Bruner was born on February 25, 1935, in Rome, Italy. He studied at the University of Rome, receiving his doctorate in chemistry in 1960 with a thesis on capillary gas chromatography and flame ionization detection. After a one-year post-doctoral fellowship at the university he joined the analytical chemistry group of the Italian National Research Council (C.N.R.). Between 1966 and 1968 he was a research associate at the Mass Spectrometry Laboratory of Massachusetts Institute of Technology, in Cambridge, MA, U.S.A. Returning to Italy he joined the Air Pollution Research Institute of the C.N.R. as a chief researcher and was promoted in 1974 to the position of the research director of the Institute. In 1970 he became "Libero Docente" in analytical chemistry at the University of Rome and in 1975 he was appointed as professor at the University of Urbino in charge of physico-chemistry and analytical chemistry. In 1960 Dr. Bruner won a national competition for full professorship. Presently he is professor at the University of Urbino and director of the Institute of Chemical Sciences of the University.

Dr. Bruner is a member of the Steering Committee of the Division of Analytical Chemistry, Società Chimica Italiana, and of the scientific committees of the Bureau Communitaire de Reference of the European Community. He is on the Editorial Advisory Boards of *Chromatographia* and *Annali di Chimica*. In 1980 he received the M.S. Tswett Memorial Medal of the All-Union Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R. Dr. Bruner is the author and coauthor of close to 100 scientific papers.

Dr. Bruner pioneered in the development of graphitized carbon blacks as stationary phases for chromatography and on the study on the chemical and physical properties of adsorbents, in the use of gas chromatography and of the combination of gas chromatography and mass spectrometry for environmental analysis.

Tsuneo Okuyama was born on May 10, 1928, in Osaka, Japan. He studied biochemistry at Osaka University receiving his B.S. in 1953, and his Dr. Sci. degree in 1959. In 1958 he joined the Department of Chemistry of Tokyo Metropolitan University as an instructor; he was appointed as an assistant professor in 1959 and then, in 1973, as a full professor of biochemistry. In 1959–1961 he was a research associate at the Foundation of Experimental Biology, in Worcester, MA, U.S.A.; in 1971 he was a visiting professor at Uppsala University, in Sweden; in 1972 a visiting scientist at the Max Planck Institute for Biochemistry, in Munich, F.R.G.; and in 1987 a visiting professor at the Department of Biotechnology of Tsinghua University, Beijing, China.

Dr. Okuyama received in 1963 the award of Toyo Rayon Science Foundation, and in 1982 the award of the Electrophoresis Society of Japan. He is the member of the editorial advisory boards of a number of Japanese scientific journals and the associate editor of *Electrophoresis*, the journal of the International Electrophoresis Society. He is currently chairman of the Division of Liquid Chromatography of the Japanese Society for Analytical Chemistry, and of various scientific committees in Japan. He is also councillor of the Electrophoresis Society of Japan, the Japanese Biochemical Society and the Japanese Neurochemical Society. He is the author and coauthor of over 250 scientific papers and a number of books.

Dr. Okuyama pioneered in the development of two-dimensional electrophoresis and its applications for the analysis of plasma proteins. He has studied the amino acid sequence in a number of proteins, and the use of isotachophoresis, isoelectric focusing and high-resolution liquid chromatography in the analysis of proteins and peptides.

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CHROMSYMP. 1514

1963–1988: TWENTY-FIVE YEARS OF INTERNATIONAL SYMPOSIA ON ADVANCES IN CHROMATOGRAPHY[a]

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SUMMARY

After outlining the origins of the International Symposia on Advances in Chromatography the past 25 Symposia are discussed briefly, and some highlights, interesting events and participants are pointed out. Finally the lessons of past events are stressed.

INTRODUCTION

Beginning with the Third Symposium, in 1965, it has always been my duty to open the first session of our symposia with a few words. This time, I intend to do something different: instead of making the customary introduction, I would like to take the participants of our 25th Symposium on a journey —a journey in time.

Almost 100 years ago, in 1895, the famous science-fiction book of H. G. Wells about the time-machine which permits one to go back and forth to any period in history or future was published. I shall borrow this machine from Wells and ask you to enter it. My purpose is to go back 25 years, to the beginning of our symposium series, to

[a] The photos used as the figures are from the personal collections of A. Zlatkis and L. S. Ettre.

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TABLE I

LOCATION AND TIME OF THE INTERNATIONAL SYMPOSIA ON ADVANCES IN CHROMATOGRAPHY, 1963–1988

No.	*Location*	*Time*
1	Houston, TX, U.S.A.	January 21–24, 1963
2	Houston, TX, U.S.A.	March 23–26, 1964
3	Houston, TX, U.S.A.	October 18–21, 1965
4	New York, NY, U.S.A.	April 3–6, 1967
5	Las Vegas, NV, U.S.A.	January 20–23, 1969
6	Miami Beach, FL, U.S.A.	June 2–5, 1970
7	Las Vegas, NV, U.S.A.	November 29–December 3, 1971
8	Toronto, Canada	April 16–19, 1973
9	Houston, TX, U.S.A.	November 4–7, 1974
10	München, F.R.G.	November 3–6, 1975
11	Houston, TX, U.S.A.	November 1–5, 1976
12	Amsterdam, The Netherlands	November 7–10, 1977
13	St. Louis, MO, U.S.A.	October 16–19, 1978
14	Lausanne, Switzerland	September 24–28, 1979
15	Houston, TX, U.S.A.	October 6–9, 1980
16	Barcelona, Spain	September 28–October 1, 1981
17	Las Vegas, NV, U.S.A.	April 5–8, 1982
18	Tokyo, Japan	April 15–17, 1982
19	Amsterdam, The Netherlands	October 3–6, 1983
20	New York, NY, U.S.A.	April 16–18, 1984
21	Oslo, Norway	June 3–6, 1985
22	Houston, TX, U.S.A.	September 15–17, 1986
23	Chiba (Tokyo), Japan	October 7–9, 1986
24	Berlin, F.R.G.	September 8–10, 1987
25	Minneapolis, MN, U.S.A.	August 28–September 1, 1988

discuss the considerations which led to the first meeting, and then to advance slowly toward the present, dealing briefly with the individual symposia (Table I), and with the people participating in them. Lest we forget: a symposium is not an object, it is a living thing, made up of people. Without their active participation, no meeting can be successful. Therefore, when reporting on the evolution of our symposium series, special emphasis must be placed on honoring those faithful chromatographers who came to every meeting, presented their newest results, and actively participated in the discussions. In this respect, it is worth mentioning that of the authors and coauthors of the papers presented at our First Symposium, in 1963, ten are also participating in the present symposium and are presenting papers here. The group photo shown in Fig. 1 was made at the Minneapolis Symposium and shows the "old timers", those who have regularly attended these symposia since the 1960s.

THE BEGINNINGS

Let us start by going back to around 1960–1962. At that time, two symposium series existed in gas chromatography, one in Europe and the other in the U.S.A. The European series, organized by the Gas Chromatography Discussion Group under the

Fig. 1. The "oldtimers" at the Minneapolis Symposium. Kneeling (left to right): C. J. W. Brooks (University of Glasgow, U.K.), E. Kováts (École Polytechnique Fédérale, Lausanne, Switzerland), A. Zlatkis (University of Houston, TX, U.S.A.) and C. G. Scott (now retired). Standing (left-to-right): W. E. Wentworth (University of Houston, TX, U.S.A.), H. J. Purnell (University College, Swansea, U.K.), D. H. Desty (now retired), Phyllis Brown (University of Rhode Island, Kingston, RI, U.S.A.), F. Bruner (University of Urbino, Italy), E. Heftmann (*Journal of Chromatography*), C. S. G. Phillips (Oxford University, U.K.), E. Bayer (Tübingen University, F.R.G.), C. L. Guillemin (Rhone Poulenc, Aubervilliers, France), M. G. Horning and E. C. Horning (both Baylor University College of Medicine, Houston, TX, U.S.A.), L. S. Ettre (Perkin-Elmer, Norwalk, CT, U.S.A.), J. A. Rijks (Eindhoven University of Technology, The Netherlands), A. Karmen (Albert Einstein College of Medicine, Bronx, NY, U.S.A.), R. E. Kaiser (Institute for Chromatography, Bad Dürkheim, F.R.G.), H. Myazaki (Nippon Kayaku Co., Tokyo, Japan), J. B. Sjövall (Karolinska Institute, Stockholm, Sweden), E. Jellum (Institute of Clinical Biochemistry, Oslo, Norway) and M. Novotny (Indiana University, Bloomington, IN, U.S.A.).

auspices of the British Institute of Petroleum, started in 1956 in London; in 1958 it was in Amsterdam, in 1960 in Edinburgh and in 1962 in Hamburg. The American series was organized by the Instrument Society of America, and was held in Lansing, MI, in 1957, 1959 and 1961. Both were highly successful series, but both were characterized by some shortcomings, and this was particularly true of the American series, *e.g.*, insufficient housing, limited participation of foreign scientists and late publication of the papers, in book form. Therefore, toward 1961–1962, a number of prominent chromatographers in the United States raised the idea of starting another symposium series, with five fundamental characteristics:

(1) The meeting program should contain contributed as well as invited papers, both from the U.S.A. and abroad. The invited papers should occupy a sizable part of the meeting and the invited scientists should represent a broad spectrum of the field.

(2) The meeting should always be accompanied by an exhibition of the latest instrumentation.

(3) The meeting should be organized in a town that is easily accessible and, preferably, in a major hotel, where everybody can be comfortably housed. Thus, it should be easy for the participants to get together and particularly meet the invited scientists.

(4) The number of organizers should be kept to a minimum and the income (*e.g.*, from the exhibition) should be used to pay the expenses of the invited key participants.

(5) The papers should be published within a very short time and *in a journal*, thus securing the broadest possible distribution.

In the course of these discussions, Albert Zlatkis, Professor at the University of Houston, Houston, TX, accepted the responsibility of setting up a symposium along these lines. Still, one needed an official sponsor, and in this respect, the University of Houston, then a fairly small school, provided a convenient background. Concerning publication, an agreement was made with L. T. Hallett, the editor of *Analytical Chemistry* that the papers would be published, after the usual peer review, in that journal as soon as possible.

THE FIRST SYMPOSIUM

The result of all of these preparations was the announcement of the First Symposium which was held in January 1963, in Houston, in the Sheraton-Lincoln Hotel. There were a total of 23 papers on the program sixteen of which (69.6%) were by American and seven (30.4%) by European authors. The scientific importance of the meeting can be indicated by a few examples: the papers of Jim Lovelock (then at the University of Houston) and Sandy Lipsky (Yale University Medical School) on ionization detectors, the fundamental discussion of Evan Horning (Baylor University College of Medicine) on the use of gas chromatography in biomedical-clinical analysis, and the very important report of István Halász (then at the University of Frankfurt, F.R.G.) on the development of support-coated open-tubular columns which represented the Ph.D. thesis of Csaba Horváth carried out in Halász' laboratory.

The meeting was a great success and this was fortified by the fact that most of the papers were published a little over two months later in the April issue of *Analytical Chemistry*. This was only possible by the close cooperation of Dr. Hallett, who participated in the symposium and managed to have most of the papers reviewed during the meeting. As emphasized in his Editorial, published in the same issue

"this symposium proved that with organization and cooperation of authors and reviewers fast publication is possible, which serves the interest of both reader and author."

After the symposium most of the foreign visitors had an opportunity to visit various American laboratories. Fig. 2 shows A. I. M. Keulemans of Eindhoven University of Technology in our laboratories, in Norwalk, CT. Lou Keulemans was one of the real pioneers in gas chromatography, first at Shell in Amsterdam, and then at Eindhoven. His book, published in 1957 by Reinhold, has served as the basic text on gas chromatography for practically everybody in my generation.

Fig. 2. Discussion at Perkin-Elmer, in 1963. Right to left: S. Ettre and A. I. M. Keulemans (Eindhoven University of Technology, The Netherlands).

ESTABLISHING THE SYMPOSIA AS A PERMANENT SERIES

The success of the first symposium encouraged Al Zlatkis to make the *Advances in Gas Chromatography* symposia a permanent series. In their organization the original goals were strictly followed: to hold truly international symposia, with a broad participation of scientists from various parts of the world reporting their latest results, to secure the rapid publication of the papers, and to provide a forum for the free and informal exchange of ideas. Since it would take too long to examine all 25 symposia from this point of view, I will just briefly summarize a few data about the first ten symposia. Table II lists the number of papers presented and their distribution according to the geographical location of their authors. As seen, the "foreign" participation generally represented 30–50% of the total number of papers. It is interesting to note that when the meeting was held overseas the American participation remained as strong as when it was held in North America: *e.g.*, in the case of the 1975 symposium, held in Germany, almost 50% of the speakers came from the U.S.A. and Canada.

But let us go back to our time-machine and briefly consider the individual symposia. I will try to pinpoint a few important papers and events and show selected photos of some of the participants.

TABLE II

DISTRIBUTION OF PAPERS PRESENTED AT THE FIRST TEN SYMPOSIA, ACCORDING TO THE GEOGRAPHICAL LOCATION OF THE AUTHORS

Symposium			*Total No. of papers*	*Papers by authors from*					
No.	*Year*	*Location*		*U.S.A. + Canada*		*Europe*		*Other locations*	
				No.	*%*	*No.*	*%*	*No.*	*%*
1	1963	U.S.A.	23	16	69.6	7	30.4	–	–
2	1964	U.S.A.	30	16	53.4	13	43.3	1	0.3
3	1965	U.S.A.	34	17	50.0	14	41.2	3	8.8
4	1967	U.S.A.	35	20	57.1	12	34.3	3	8.6
5	1969	U.S.A.	52	37	71.2	13	25.0	2	3.8
6	1970	U.S.A.	53	35	66.0	18	34.0	–	–
7	1971	U.S.A.	46	28	60.9	16	34.8	2	4.3
8	1973	Canada	66	34	51.5	28	42.4	4	6.1
9	1974	U.S.A.	61	38	62.3	15	24.6	8	13.1
10	1975	F.R.G.	59	27	45.8	29	49.1	3	5.1

THE EARLY SYMPOSIA

The Second and Third Symposia were still held in Houston, in 1964 and 1965. Many important papers were presented at the Second Symposium; for example, J. E. Lovelock, G. R. Shoemake and A. Zlatkis described an improved ionization cross-section detector, and Arthur Karmen (then at Johns Hopkins University School of Medicine) discussed for the first time the possibility of making the flame-ionization detector selective for halogen- and phosphorus-containing compounds. Roy Teranishi and T. R. Mon (U.S.D.A. Western Regional Research Laboratory) reported on long, open-tubular columns with 0.50 and 0.75 mm I.D. for use in combined gas chromatography–mass spectrometry systems, while Fabrizio Bruner and Gianpaulo Cartoni (then at the University of Naples, Italy) disclosed for the first time the separation of cyclohexane and deuterocyclohexane in open-tubular columns. An important presentation was that of Ervin Kováts (then at the Federal Technical University, in Zürich) on the retention index system. Lou Keulemans showed an interesting gadget for demonstrating retention time shifting as a function of relative retention of two closely located peaks. Larry Hallett liked it so much that he used it for the title page of the July 1964 issue of *Analytical Chemistry* in which the symposium papers were published. An important participant at the 1964 Symposium was A. V. Kiselev of Lomonosov State University of Moscow (Fig. 3), probably the most outstanding scientist of our generation in the field of adsorption. He presented two papers at the meeting.

With the Third Symposium we started to outgrow the facilities provided by *Analytical Chemistry*. Therefore, the papers were divided between two journals and published in the February 1966 issue of *Analytical Chemistry* and the January, February and March 1966 issues of the *Journal of Gas Chromatography*. Also,

Fig. 3. A. V. Kiselev (Lomonosov State University of Moscow, U.S.S.R.), lecturing at the 1964 Houston Symposium.

a separate Symposium Proceedings Volume was provided by the Preston Technical Abstracts Co., the publisher of the *Journal of Gas Chromatography*. Among the gas chromatography papers presented, three may be mentioned here: Victor Pretorius (University of Pretoria, South Africa) discussed the possibilities of turbulent-flow gas chromatography, a subject which has recently become of interest again: O. L. Hollis (Dow Chemical Co., Freeport, TX, U.S.A.) presented a detailed report on the porous polyaromatic polymer beads; and S. S. Brody and J. E. Chaney (Melpar Inc., Falls Church, VA, U.S.A.) described the flame-photometric detector selective for sulfur- and phosphorus-containing compounds.

EXPANDING THE SCOPE OF THE SYMPOSIA

The 1965 Symposium was very important in the evolution of chromatography for a very special reason. This was the period when a number of researchers were engaged in investigations aimed at the "modernization" of liquid chromatography, in three fields: theory, column technology and instrumentation. Among others they tried to adapt the flame-ionization detector to liquid chromatography, and great hopes were placed in the so-called moving-chain flame-ionization detector. Three versions of this detector were described at this meeting, by J. E. Stouffer (Baylor University of Medicine), Arthur Karmen, and E. Haahti (University of Turku, Finland). According to the Foreword to the Symposium Proceedings

> "the reason for the inclusion of these papers in the program ... was that these new developments in liquid chromatography are directly related to achievements in gas chromatography. It is our belief that the unparalleled development of gas chromatography in the last ten years will also permit in the near future a significant breakthrough in liquid chromatography and that these two fields should not be treated separately but rather as interrelated disciplines."

In addition, a special discussion session was held at the 1965 symposium on the potentialities of modern liquid chromatography. As far as I can remember this was the first discussion of this subject at a major chromatography meeting.

The correctness of the prediction in the Foreword of the Third Symposium was evident from the Fourth Symposium, held in 1967. There, four papers were included in the program on various aspects of liquid chromatography and a special informal discussion session further explored the possibilities. Then, two years later, by the Fifth Symposium, held in 1969 in Las Vegas, liquid chromatography had already become a full-fledged partner to gas chromatography. The situation was best characterized by Al Zlatkis in the Foreword of the Symposium Proceedings:

"During the past few years, artificial classification barriers, which have been dividing various types of chromatography have started to erode. Samples considered to be specific for a particular type of chromatography are now being subjected to other techniques, and theories which at the early stage of development were treated separately are now presently going through a unifying approach. It is significant to note that parallel to this development, scientists, who during the genesis of gas chromatography became identified with this technique, are now active in the other branches of chromatography.

This trend started about 1965 ... and became more evident at the last Symposium held in March 1967 ... Today, however, it is no longer a trend but an established fact."

Indeed, this fact was best illustrated by the sixteen papers dealing with liquid chromatography representing 30.8% of all the papers presented at the 1969 Symposium. Due to the importance of this symposium as the first major international forum where a large number of papers reported on newest developments in liquid chromatography, their titles and authors are listed in Table III. Fig. 4 is a snapshot from this meeting.

To emphasize further that gas and liquid chromatography represent interrelated disciplines, the title of the symposium series was changed: starting with the 1969 meeting, they are called the symposia on *Advances in Chromatography*. At that time this was a unique decision: the European biannual symposium series did not delete "gas" from its title until 1974, although a limited number of papers on liquid chromatography (considered a "related technique") were in its program since 1966.

The publication of the large number of papers presented at the 1969 Symposium represented a problem and they had to be distributed throughout almost all the 1969 issues of the *Journal of Chromatographic Science* (the new name of the *Journal of Gas Chromatography*). However, they were also available in a limited-edition bound volume, published by the Preston Technical Abstracts Company. The same was true of the 1970 Symposium: these papers were published in eight issues of that journal, having again a bound Proceedings Volume available.

In 1970, we went to the Fountainebleau Hotel, in Miami Beach, where a few years before *Goldfinger*, the James Bond movie, was made. Fig. 5 was taken in that hotel. My personal story associated with this, our Sixth Symposium, is related to my opening remarks. By mistake, I picked up and almost started to read the speech of Teddy Kollek, the mayor of Jerusalem, given the night before, the manuscript of which he left on the speaker's stand...

As a continuation of the Las Vegas Meeting, a number of the papers presented in Miami dealt with modern liquid chromatography. In addition, four papers reported

TABLE III

PAPERS ON MODERN LIQUID CHROMATOGRAPHY PRESENTED AT THE *FIFTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY, JANUARY 20–23, 1969, LAS VEGAS, NV, U.S.A.*[a]

Theory and practice

Cs. Horváth and S. R. Lipsky (Physical Sciences Section, Yale University School of Medicine, New Haven, CT, U.S.A.): Column design in high-pressure liquid chromatography.

R. P. W. Scott and J. G. Lawrence (Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire, U.K.): The effect of temperature and moderator concentration on the efficiency and resolution of liquid chromatography columns.

J. F. K. Huber (Analytical Chemistry Laboratory, University of Amsterdam, Amsterdam, The Netherlands): High efficiency, high speed liquid chromatography in columns.

L. R. Snyder and D. L. Saunders (Research Department, Union Oil Company of California, Brea, CA, U.S.A.): Optimized solvent-programming for separation of complex samples by liquid-solid adsorption chromatography in columns.

R. J. Maggs (Pye Unicam Ltd., Cambridge, U.K.): The role of temperature in liquid-solid chromatography: some practical considerations.

Column technology

I. Halász and P. Walking (Institute of Physical Chemistry, Unversity of Frankfurt, F.R.G.): Different types of packed columns in liquid-solid chromatography.

S. T. Sie and N. van den Hoed (Koninklijke/Shell Laboratory, Shell Research NV, Amsterdam, The Netherlands): Preparation and performance of high-efficiency columns for liquid chromatography.

J. J. Kirkland (Industrial and Biochemical Department, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, DE, U.S.A.): High-speed liquid chromatography with controlled surface porosity supports.

K. J. Bombaugh, W. A. Dark and R. F. Levangie (Waters Associates, Inc., Framingham, MA, U.S.A.): High resolution steric chromatography.

J. C. Giddings, M. N. Myers and J. W. King (Department of Chemistry, University of Utah, Salt Lake City, UT, U.S.A.): Dense gas chromatography at pressures to 2000 atmospheres.

Detectors and liquid chromatographs

J. F. K. Huber (Analytical Chemistry Laboratory, University of Amsterdam, Amsterdam, The Netherlands): Evaluation of detectors for liquid chromatography in columns.

M. N. Munk and D. N. Raval (Varian Aerograph, Walnut Creek, CA, U.S.A.): Flow sensitivity of the micro-adsorption detector.

T. W. Smuts, F. A. van Niekerk and V. Pretorius (Department of Physical and Theoretical Chemistry, University of Pretoria, Pretoria, South Africa): Modified micro-adsorption detector.

T. W. Smuts, D. J. Solms, F. A. van Niekerk and V. Pretorius (Department of Physical and Theoretical Chemistry, University of Pretoria, Pretoria, South Africa): A splitter inlet system for liquid chromatography.

H. Coll, H. W. Johnson, Jr., A. G. Polgar, E. E. Seibert and F. H. Stross (Shell Development Company, Emeryville, CA, U.S.A.): The belt detector in the quantitative analysis of polymers by gel permeation chromatography.

H. Felton (Development Department, Instrument Products Division, E. I. du Pont de Nemours & Co., Experimental Station, Wilmington, DE, U.S.A.): Performance of components of a high-pressure liquid chromatography system.

[a] The papers were published in Vol. 7 (1969) of the *Journal of Chromatographic Science*. The papers were also published in a limited-edition bound volume [*Advances in Chromatography —Fifth International Symposium, Las Vegas, NV, January 20–23, 1969*, edited by Albert Zlatkis; Preston Technical Abstracts Co., Evanston, IL, 1969] serving as the proceedings of the symposium.

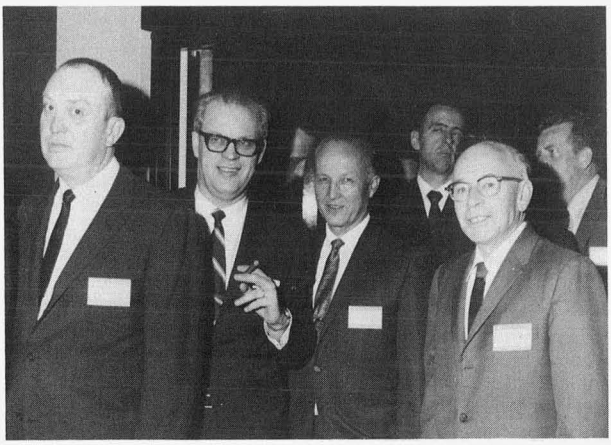

Fig. 4. 1969 Las Vegas Symposium. First row left to right: C. Merritt Jr. (U.S. Army Pioneering Research Laboratory, Natick, MA, U.S.A.), L. S. Ettre, F. Eggertsen and A. G. Polgar (both Shell Development Co.); in the background, between Eggertsen and Polgar, L. R. Snyder (then Union Oil of California); on the right, R. A. Keller, the editor of the *Journal of Chromatographic Science*.

Fig. 5. 1970 Miami Beach Symposium. Left to right: J. E. Lovelock, A. J. P. Martin (then with the University of Houston) and A. Zlatkis.

on new approaches in thin-layer chromatography; starting with this meeting, thin-layer chromatography became the third chromatographic technique to be included in the scope of the symposia.

In gas chromatography, three papers dealt with its combination with pyrolysis: among them was that of Willy Simon of the Federal Technical Institute, in Zürich, who reported on the theory and practice of Curie-point pyrolysis. A number of papers dealt with flavor analysis; *e.g.*, H. M. Liebich, E. Bayer and A. Zlatkis (University of Houston) reported on investigations of various cheeses. K. Grob and G. Grob (University of Zürich) reported on trace analysis utilizing 0.32–0.35-mm I.D. glass capillary columns. As emphasized in the Foreword to the Sixth Symposium Proceedings, the success of gas chromatography was "crowned by its application in the technological achievement of the century: the start of exploration of the universe by mankind". This was detailed in an elaborate paper coauthored by John Oro and his colleagues of the Department of Biophysical Sciences and Al Zlatkis of the Department of Chemistry (both of the University of Houston) and Emmanuel Gil-Av of the Weizmann Institute of Sciences (Rehovot, Israel), then a visiting scientist at Houston. They reported on their gas chromatography–mass spectrometry studies of the lunar samples from the Sea of Tranquility.

In 1971, we went back again to Las Vegas. Our Seventh Symposium was characterized by an unusually large number of papers on detectors and pyrolysis–gas chromatography, representing 19.6 and 10.9%, respectively, of all papers presented. From the former group, the paper by Jim Lovelock on the new mode of operation (constant current) of the electron-capture detector, extending its linear range, while from the latter group, the report by R. L. Levy (McDonnell Douglas Corp., St. Louis, MO, U.S.A.) on the possibility of using a focused laser beam as the heat source deserve special mention. Another important paper, by Ted Adlard (Shell Research, Chester, U.K.), dealt with the identification of hydrocarbon pollutants of seas and beaches by capillary gas chromatography and the simultaneous use of flame-ionization and sulfur-specific flame-photometric detectors. The newest results in the evolution of modern liquid chromatography, dealing with theory, columns, instrumentation and applications, were the subject of ten papers (21.7%).

The 1971 Symposium also served an unique purpose. Patrick Howard, a graduate student at the University of Houston, tried for months to defend his thesis in Houston, but the faculty could never get his entire committee together. However, when it was realized that all committee members would be in Las Vegas during our Symposium he finally had the opportunity to defend his thesis. Fig. 6 shows a photo of this memorable occasion. Other photos taken at the 1971 Las Vegas Symposium are shown in Figs. 7 and 8.

The papers presented at the 1971 Symposium were published in the December 1971 and January 1972 issues of *Analytical Chemistry* and also collected in a separate book, available at the Symposium. 1971/1972 represented the thirtieth anniversary of the classical paper on partition chromatography by Martin and Synge, published in the December 1941 issue of the *Biochemical Journal*, and the twentieth anniversary of the original publication on gas–liquid partition chromatography by James and Martin, published in 1952 in the same journal. To commemorate these two milestone papers, they were reprinted in the Symposium Proceedings.

Fig. 6. 1971 Las Vegas Symposium: Patrick Howard defending his Ph.D. Thesis. Sitting, left to right: John Oro (University of Houston), E. Gil-Av (Weizmann Institute, Rehovot; then guest professor at Houston), W. E. Wentworth, John Bear, Wolfgang Parr, Al Zlatkis and R. Halpern (all of the University of Houston).

VISITING CANADA

In 1973 we ventured out of the U.S.A. for the first time: the Eighth Symposium was held in Toronto, Canada. This was also the second time we had visitors from the Soviet Union, now in the person of Karl Ivanovich Sakodynskii (Fig. 9), a pioneer in

Fig. 7. 1971 Las Vegas Symposium. Left to right: E. Bayer (Tübingen University, F.R.G.) and István Halász (then the University of Frankfurt, F.R.G.).

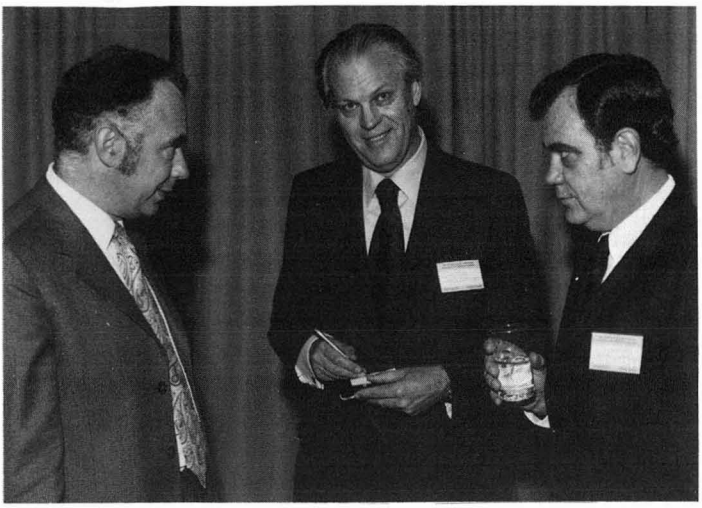

Fig. 8. 1971 Las Vegas Symposium. Left to right: David R. Deans (I.C.I., Petrochemical Division, Billingham, U.K.), L. S. Ettre and J. E. Baudean (Perkin-Elmer, Norwalk, CT, U.S.A.).

the use of gas chromatography on a semi-industrial scale. The scientific program contained, among others, one paper by Evan and Marjorie Horning and their coworkers describing a new mass spectrometer with external ionization source at atmospheric pressure, and another paper on the use of thermostable glass open-tubular columns in biomedical analysis; Al Zlatkis and coworkers presented a detailed report on the analysis of metabolitic profiles in urines by capillary gas chromato-

Fig. 9. 1973 Toronto Symposium. Left to right: E. C. Horning (Baylor University College of Medicine), K. I. Sakodynskii (Karpov Institute, Moscow, U.S.S.R.) and Al Zlatkis.

graphy–mass spectrometry; David Deans (I.C.I., Billingham, U.K.) described how the selectivity of a two-column system could be modified by changing the individual gas velocities; and Egil Jellum (Institute of Clinical Biochemistry, University of Oslo) presented his first detailed paper (followed by many additional reports at subsequent symposia) on the possibility of identifying metabolic disorders by the combined use of gas chromatography and mass spectrometry. Among the papers on liquid chromatography we may mention the report of Ray Scott (now Hoffmann-La Roche, Nutley, NJ, U.S.A.) on the selection of the most suitable solvents for gradient elution, and the paper of Phyllis Brown (University of Rhode Island) describing the use of liquid chromatography in the investigation of *in vitro* reactions of human erythrocytes.

ESTABLISHING THE M. S. TWETT CHROMATOGRAPHY AWARD

In 1974, we went back to Houston: our Ninth Symposium was held there in November. This meeting was very important for two reasons.

The first concerns the publication of the papers. Until now, the papers of the symposia were published in *Analytical Chemistry* and the *Journal of Gas Chromatography* which later became the *Journal of Chromatographic Science*. However, we simply outgrew these journals. Fortunately, the *Journal of Chromatography* just started to publish entire symposia as separate volumes and agreed to cover our meetings, effective with the Ninth Symposium. In addition to the publication of all the submitted papers as a separate journal volume, a bound copy was also prepared which, for a few years, was already available at the symposium. The Proceedings of the Ninth Symposium was the first handled by this journal: it is a massive, 772-page long volume. From then on, the papers presented at each symposium were handled in this way.

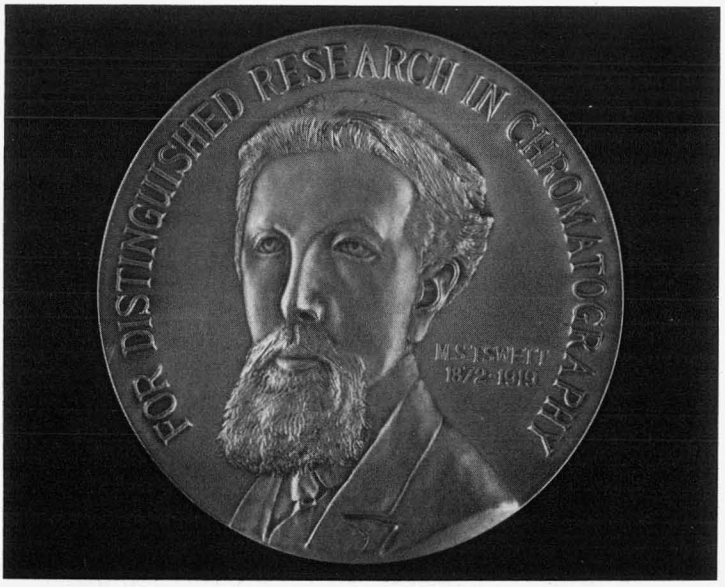

Fig.10. The M. S. Tswett Chromatography Award medal.

I want to take this opportunity to thank Elsevier Science Publishers and the journal editors, Michael Lederer and Erich Heftmann, for their cooperation in making this possible.

The second important event connected with the 1974 Symposium was the establishment of the M. S. Tswett Chromatography Award. It had been felt for some time, that, in addition to the American Chemical Society Award in Chromatography given to one person per year, there should be another special recognition to be given more informally to both pioneers and younger researchers in our field. After much planning, the M. S. Tswett Chromatography Award, consisting of a medal (Fig. 10) and a scroll, was established. It was first given at our 1974 Symposium to five pioneers: Erika Cremer (University of Innsbruck, Austria), who carried out gas-adsorption chromatography as early as 1944; Denis Desty (British Petroleum), who was the first to apply the original work of James and Martin on gas–liquid partition chromatography; Lou Keulemans and A. V. Kiselev, whose activities I have already mentioned, and A. J. P. Martin, the inventor of partition chromatography. The photos in Figs. 11 and 12 were made at the award banquet.

Since 1974, the Tswett Medal has been presented at every symposium to selected scientists who excelled in our field. The list of 51 award winners (Table IV) from 14 countries truly represents a Who-is-Who in chromatography.

Looking over the subjects of the scientific papers presented at the 1974 Symposium, we can identify two particularly strong fields. The first concerns glass capillary columns. Present-day chromatographers tend to forget that as recently as 15 years ago, even these columns met with only limited acceptance; *e.g.*, the paper of

Fig. 11. 1974 Houston Symposium. D. H. Desty (British Petroleum) speaking at the Award Banquet.

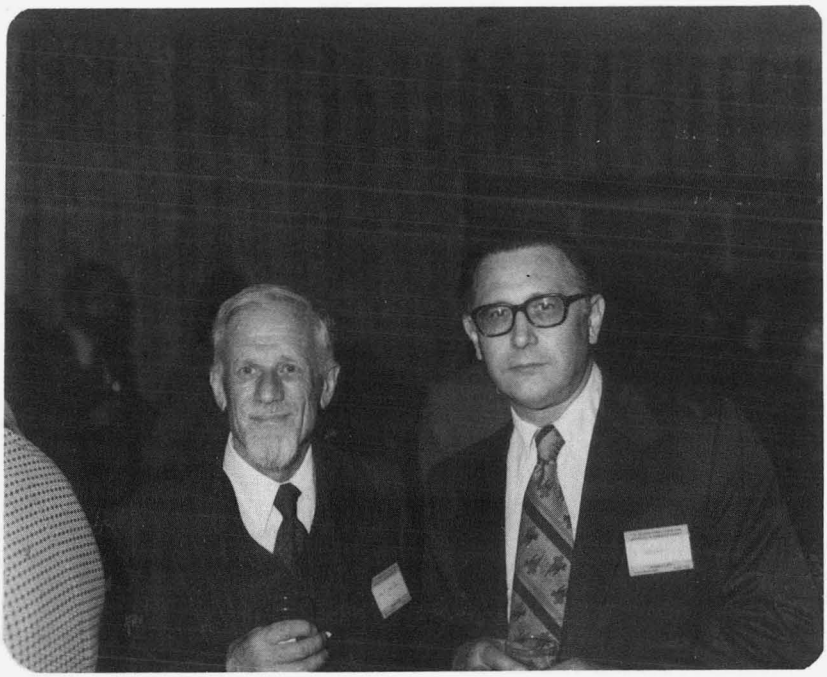

Fig. 12. 1974 Houston Symposium. Left to right: H. F. Walton (University of Colorado) and Csaba Horváth (Yale University).

Schomburg states that "the application of glass capillaries ... seems to be unpopular", and he lists four reasons for it. Therefore, the 1974 Symposium deserves special mention as a milestone in this evolution, particularly on the basis of three papers presented in Houston.

The first was the above-mentioned presentation by Gerhard Schomburg (Max-Planck-Institut für Kohlenforschung, Mülheim, F.R.G.) in which he discussed in detail the questions associated with the preparation and performance of glass open-tubular columns. In addition, the paper by Gábor Alexander (Hungarian Academy of Sciences, Budapest, Hungary), reported on the characteristics of the surfaces of treated glass tubes, while Evan Horning and coworkers described a special technique for producing thermally stable glass capillary columns, coated with a polar phase.

The second particularly noteworthy field dealt with was biomedical applications of chromatography. Among the papers presented, Phyllis Brown described the analysis of purine and pyrimidine bases by liquid chromatography while Charles Brooks (University of Glasgow, U.K.) reported on studies of urinary metabolites by gas chromatography–mass spectrometry.

Finally, the third important field represented was modern chromatography instrumentation. Evan and Marjorie Horning reported on liquid chromatography–mass spectrometry systems: Jim Lovelock presented a theoretical model of the electron-capture detector and described a new coulometric detector; Marty Hartigan

and John Purcell (Perkin-Elmer, Norwalk, CT, U.S.A.) with Milton Lee and Milos Novotny (Indiana University, Bloomington, IN, U.S.A.) presented the definitive paper on the performance of a nitrogen-sensitive detector for gas chromatography; and Stuart Cram (then at the U.S. National Bureau of Standards) discussed in detail the potentialities of fluidic logic sampling systems for gas chromatography.

VENTURING INTO EUROPE

1975 represented a milestone in the evolution of our symposia. This was the first time we crossed the Ocean; our Tenth Symposium was held in Munich, F.R.G. We again honored five pioneers of chromatography by the Tswett Award (Fig. 13); it was presented to Gerhard Hesse, professor at the University of Erlangen and the great old man of chromatography in Germany, who was one of the first in the thirties to apply chromatography to the analysis of complicated mixtures of natural substances, to Evan Horning, who revolutionized biochemical analysis, to Jaroslav Janák (Institute of Instrumental Analytical Chemistry of the Czechoslovak Academy of Sciences, in Brno) who in the early 1950s developed a gas chromatograph for the analysis of gaseous mixtures, to Jim Lovelock, the inventor of the electron-capture and other ionization detectors, and to Courtenay Phillips of Oxford University whose activities in gas chromatography began in 1946. The award winners were honored by a reception and Figs. 14 and 15 are snapshots from it.

In spite of the fact that now the meeting was held in Europe, a fairly large American contingent participated in it: 27 papers representing 45.8% of the total were presented by American authors (see Table II).

The papers presented covered a very broad field; just a few examples are given here. Howard Purnell (University College of Swansea, Wales) presented the theory of mixed stationary phases in gas chromatography and the optimization of their composition via the so-called window-diagrams; István Halász (then at the University of Saarbrücken) discussed the ultimate limits in high-pressure liquid chromatography; Victor Pretorius described techniques for growing whiskers on the inside wall of glass capillary tubes thus increasing the coated surface; Bruno Kolb (Bodenseewerk Perkin-Elmer & Co., Überlingen, F.R.G.) presented the theory and practice of headspace gas chromatography for the characterization of non-ideal solutions; Georges Guiochon (then at the École Polytechnique, Palaiseau, France) critically evaluated the syringe pumps used in liquid chromatography; and Gerhard Schomburg described in detail the technique of multi-dimensional capillary gas chromatography, illustrating its applications. Although it was not presented at the symposium, the journal issue and the proceedings also contained a long article on the development of gas chromatography, since the 1920s.

From 1975 on, our symposia were alternately organized in the U.S.A. and overseas. Thus the Eleventh Symposium was held in November 1976 in Houston. The Tswett Chromatography Award was presented to Marcel Golay of Perkin-Elmer, for the invention of open-tubular columns, to Georges Guiochon, for his many contributions to the progress of gas and liquid chromatography, to Tony James (now at Unilever, Sharnbrook, U.K.) who, with Archer Martin, first described gas–liquid partition chromatography in 1952, to Edgar Lederer (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France) who, in 1930–1931 reintroduced

TABLE IV

WINNERS OF THE M. S. TSWETT CHROMATOGRAPHY AWARD (1974–1988)

Name	*Affiliation**	*Year*	*Citation (fields)*
E. Bayer	University of Tübingen, F.R.G.	1986	Liquid chromatography; chiral phases; combination of liquid chromatography and nuclear magnetic resonance spectroscopy
P. R. Brown	University of Rhode Island, RI, U.S.A.	1988	Liquid chromatography in biomedical research and clinical analysis
F. Bruner	University of Urbino, Italy	1988	Capillary adsorption columns; gas chromatography–mass spectrometry in environmental analysis
C. A. Cramers	University of Technology, Eindhoven, The Netherlands	1986	Small-diameter open-tubular columns in gas chromatography; gas chromatography–mass spectrometry; high-speed chromatography
E. Cremer	University of Innsbruck, Austria	1974	Pioneering in gas chromatography; use of gas chromatography for physicochemical measurements
D. H. Desty	British Petroleum, Sunbury-on-Thames, U.K.	1974	Pioneering work in gas chromatography
L. S. Ettre	Perkin-Elmer Corp., Norwalk, CT, U.S.A.	1978	Open-tubular columns in gas chromatography
P. Flodin	Chalmers University of Technology, Göteborg, Sweden	1979	Gel-filtration chromatography
J. C. Giddings	University of Utah, Salt Lake City, UT, U.S.A.	1978	Theory of chromatography
M. J. E. Golay	Perkin-Elmer Corp., Norwalk, CT, U.S.A.	1976	Invention of open-tubular columns for gas chromatography; theory of chromatography
G. Guiochon	École Polytechnique, Palaiseau, France	1976	Gas and liquid chromatography
I. Halász	University of Saarbrücken, F.R.G.	1980	Gas and liquid chromatography
S. Hara	Tokyo College of Pharmacy, Japan	1986	Liquid chromatography; thin-layer chromatography
H. Hatano	University of Kyoto, Japan	1982	Liquid chromatography
G. Hesse	University of Erlangen, F.R.G.	1975	Pioneering work in liquid chromatography
E. C. Horning	Baylor University of Medicine, Houston, TX, U.S.A.	1975	Applications of gas and liquid chromatography in biochemical and clinical analysis
M. G. Horning	Baylor University of Medicine, Houston, TX, U.S.A.	1987	Applications of gas and liquid chromatography in analytical biochemistry and pharmacology
Cs. Horváth	Yale University, New Haven, CT, U.S.A.	1980	Pioneering in modern liquid chromatography
N. Ikekawa	Tokyo Institute of Technology, Japan	1982	Applications of chromatography to the analysis of natural substances
D. Ishii	Nagoya University, Japan	1987	Small-diameter columns in liquid chromatography
A. T. James	Unilever, Sharnbrook, U.K.	1976	Pioneering in gas–liquid partition chromatography
J. Janák	Institute of Instrumental Analytical Chemistry, Brno, Czechoslovakia	1975	Gas-adsorption chromatography
E. Jellum	Institute of Clinical Biochemistry, Oslo, Norway	1983	Biomedical applications of chromatography
B. L. Karger	Northeastern University, Boston, MA, U.S.A.	1986	Liquid chromatography in biological sciences; biopolymer separations

A. Karmen	Albert Einstein University of Medicine, Bronx, NY, U.S.A.	1982	Applications of gas and liquid chromatography in clinical analysis
A. I. M. Keulemans	University of Technology, Eindhoven, The Netherlands	1974	Pioneering work in gas chromatography
A. V. Kiselev	State University of Moscow, U.S.S.R.	1974	Adsorption and chromatography
J. H. Knox	University of Edinburgh, U.K.	1985	Modern liquid chromatography
E. sz. Kováts	École Polytechnique Fédérale, Lausanne, Switzerland	1977	Gas chromatography; invention of the retention index system
E. Lederer	Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France	1976	Pioneering in liquid chromatography
M. L. Lee	Brigham Young University, Provo, UT, U.S.A.	1984	Open-tubular columns in gas chromatography; capillary supercritical-fluid chromatography
A. Liberti	University of Rome, Italy	1981	Gas chromatography; environmental analysis
S. R. Lipsky	Yale University Medical School, New Haven, CT, U.S.A.	1982	Gas chromatography; gas chromatography–mass spectrometry; open-tubular columns
J. E. Lovelock	Consultant, Bowerchalke, U.K.	1975	Ionization detectors for gas chromatography; invention of the electron-capture detector
K. Macek	Institute of Physiology, Charles University, Prague, Czechoslovakia	1985	Paper chromatography; biochemical applications of chromatography
A. J. P. Martin	University of Sussex, Brighton, U.K. and University of Houston, TX, U.S.A.	1974	Invention of partition chromatography
H. Miyazaki	Nippon Kayaku Co., Tokyo, Japan	1986	Chromatography and isotachophoresis of biologically important substances
M. Novotny	Indiana University, Bloomington, IN, U.S.A.	1984	Capillary columns in gas and liquid chromatography; supercritical-fluid chromatography
T. Okuyama	Tokyo Metropolitan University, Japan	1988	Two-dimensional electrophoresis; biochemical analysis
C. S. G. Phillips	Oxford University, U.K.	1975	Pioneering work in gas chromatography
C. F. Poole	Wayne State University, Detroit, MI, U.S.A.	1985	Selective detection; new stationary phases
J. O. Porath	University of Uppsala, Sweden	1979	Gel-filtration and affinity chromatography
V. Pretorius	University of Pretoria, South Africa	1976	Pioneering work in gas and liquid chromatography
J. H. Purnell	University College, Swansea, U.K.	1977	Pioneering work in gas chromatography
K. I. Sakodynskii	Karpov Institute of Physical Chemistry, Moscow, U.S.S.R.	1981	Preparative gas chromatography; cooperation between scientists from different countries
G. Schomburg	Max Planck Institut für Kohlenforschung, Mülheim, F.R.G.	1983	Gas and liquid chromatography; multidimensional chromatography
R. P. W. Scott	Hoffmann-La Roche, Nutley, NJ, U.S.A.	1978	Gas and liquid chromatography
R. E. Sievers	University of Colorado, Boulder, CO, U.S.A.	1981	Use of gas chromatography in environmental analysis
J. B. Sjövall	Karolinska Institute, Stockholm, Sweden	1987	Applications of chromatography in biochemical analysis and medicine
A. A. Zhukhovitskii	Steel & Alloys Institute, Moscow, U.S.S.R.	1977	Pioneering work in gas chromatography
A. Zlatkis	University of Houston, TX, U.S.A.	1983	Open-tubular columns in gas chromatography; trace analysis

* Affiliation when receiving the award.

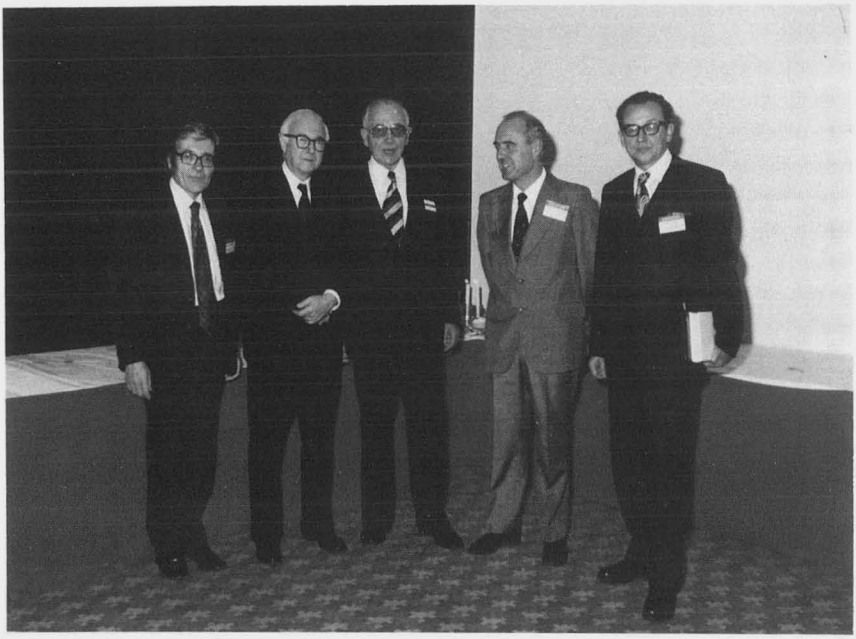

Fig. 13. 1975 Munich Symposium: the Tswett Chromatography Award winners. Left to right: J. E. Lovelock, E. C. Horning (Baylor University College of Medicine), G. Hesse (Erlangen University), C. S. G. Phillips (Oxford University) and J. Janák (Czechoslovak Academy of Sciences, Brno).

Fig. 14. 1975 Munich Symposium. Left to right: L. S. Ettre, Phyllis R. Brown (University of Rhode Island), Erika Cremer (University of Innsbruck) and J. F. K. Huber (then with the University of Amsterdam).

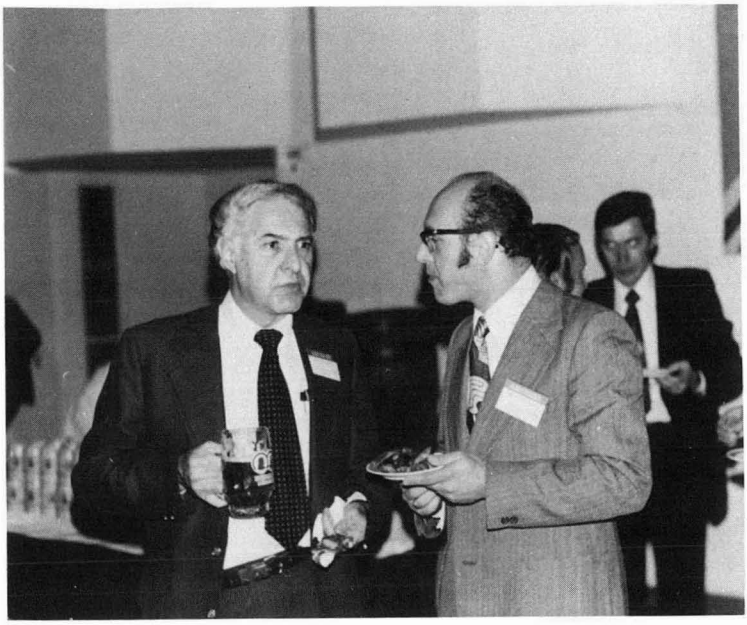

Fig. 15. 1975 Munich Symposium: S. R. Lipsky (Yale University Medical School) (left) and Gábor Alexander (Hungarian Academy of Sciences, Budapest).

Fig. 16. 1977 Amsterdam Symposium: reception in the Rijksmuseum. Right to left: Dr. & Mrs. A. Zlatkis, Dr. & Mrs. G. Dijkstra (Utrecht University).

Fig. 17. 1977 Amsterdam Symposium: J. H. Purnell (University College at Swansea, U.K.) receiving the Tswett Award.

Fig. 18. 1977 Amsterdam Symposium: A. A. Zhukhovitskii (Steel and Alloys Institute, Moscow, U.S.S.R.) receiving the Tswett Award.

Tswett's chromatographic method and applied it to the investigation of natural substances, and to Victor Pretorius, for his numerous contributions to the development of chromatography. Among the 63 papers included in the Proceedings at least three should be mentioned: Ervin Kováts' (now at the École Polytechnique Fédérale de Lausanne) description of a tailor-made C_{87} hydrocarbon as a possible standard non-polar stationary phase for gas chromatography; the combined report of M. Stafford and M. G. Horning (Baylor College of Medicine) and Al Zlatkis (University of Houston) on the determination of profiles of volatile metabolites in body fluids; and Arthur Karmen's (now at Albert Einstein College of Medicine) discussion on the use of chemical-ionization mass spectrometry for the rapid assay of drugs in serum.

The Twelfth Symposium, in 1977, was held in Amsterdam, the Netherlands. Professor Dijkstra of the State University in Utrecht helped us in the organization (Fig. 16). The Tswett Award was given to Ervin Kováts for the development of the retention index system; to Howard Purnell, for his detailed studies related to the thermodynamics of chromatography; and to A. A. Zhukhovitskii, the great old man of chromatography in the U.S.S.R. Figs. 17 and 18 were made during the ceremonies. The symposium proceedings consisted of 68 papers 32 of which (47.1%) dealt with liquid column and thin-layer chromatography.

The Thirteenth Symposium was held in 1978, in St. Louis, MO. The Tswett Chromatography Award was given to three scientists: L. S. Ettre (Fig. 19), Calvin Giddings of the University of Utah, for his pioneering work in developing a unified theory of chromatography, and Ray Scott (Hoffmann-La Roche) for his continuous contributions to chromatography during the last quarter century. In the scientific

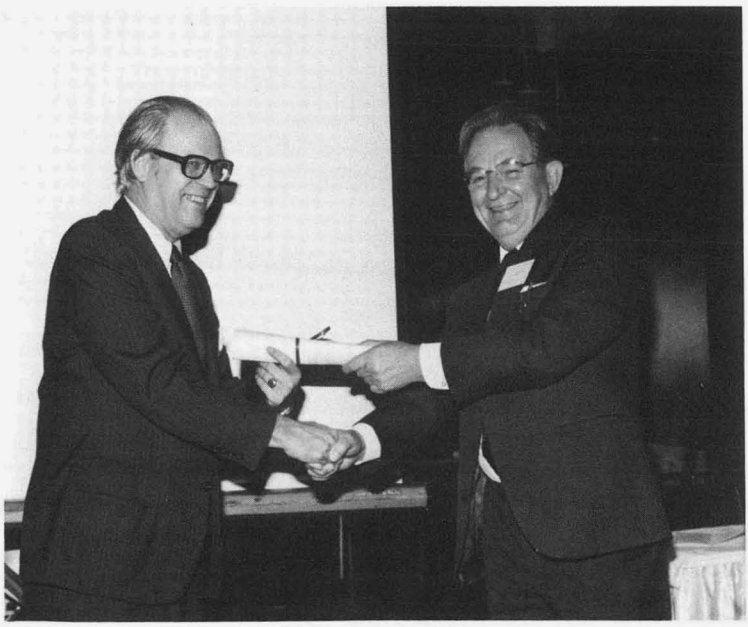

Fig. 19. 1978 St. Louis Symposium: L. S. Ettre receiving the Tswett Award from D. H. Desty, acting as the Master of Ceremonies.

program, Jim Lovelock continued the discussion of the theory and practice of the electron-capture detector (the first part was presented at the 1974 Symposium); Csaba Horváth (Yale University) enhanced the theory of hydrophobic interactions in reversed-phase liquid chromatography, Cal Giddings reported on newest results in field-flow fractionation; Arthur Karmen outlined the possibility of specific detection of nitrogen-containing compounds in gas chromatography by converting them to ammonia and using fluorescent detection; and Daido Ishii (Nagoya University, Japan) presented a detailed report on open-tubular microcapillary liquid chromatography with bonded stationary phase. A superb wine-tasting party at one of the evenings proved that we still like to adhere to the original meaning of the Greek word *symposion* (according to *Webster's New Collegiate Dictionary*, "a drinking party", "a social gathering at which there is free interchange of ideas"); Fig. 20 was made on this occasion.

In 1979 we again crossed the Ocean, organizing our Fourteenth Symposium in Lausanne, Switzerland, with the help of Ervin Kováts and the École Polytechnique Fédérale de Lausanne (EPFL), the downtown auditorium of which presented an excellent location for the sessions and the exhibition; Fig. 21 was made at the opening reception. Per Flodin (Chalmers University of Technology, Göteborg) and Jerker Porath (Uppsala University) were honored by the Tswett Chromatography Award, for their pioneering work in the development of gel-filtration chromatography. This meeting was particularly memorable because it was the first time that chromato-

Fig. 20. 1978 St. Louis Symposium: wine tasting party. Left to right: R. P. W. Scott (Hoffmann-La Roche), S. R. Lipsky (Yale University Medical School) and R. Kaiser (Institute for Chromatography, Bad Dürkheim, F.R.G.).

Fig. 21. 1979 Lausanne Symposium. Left to right: Bernard Vittoz, president of EPFL, R. E. Sievers (University of Colorado), E. sz. Kováts (EPFL), Philippe Javet (EPFL) and Mrs. Sievers.

graphers from the People's Republic of China could attend: Fig. 22 shows the two delegates. A large number of papers were presented at the symposium: the Proceedings Volume contains 77 papers, 27 of which (35.1%) deal with biomedical and environmental applications. This meeting was held at a cross-road of chromato-

Fig. 22. 1979 Lausanne Symposium: guests from the People's Republic of China. From the right: Prof. Lu Peichang (Dalian) and Dr. Deng Liru (Beijing) with L. S. Ettre.

graphy: the first disclosure about fused-silica capillary columns was made in the spring of 1979, at the Hindelang Symposium, and thus by September more data were available about them. There was not enough time to schedule formal papers on these columns in the program of the Lausanne Symposium, but Sandy Lipsky reported on his newest results, and there was a lot of discussion about the merits of fused-silica capillaries.

THE 1980s

We are now close to the present and thus, the time machine is accelerating: I will report only briefly about the symposia in this decade; their scientific merits will have to be assessed by the next generation.

Our Fifteenth Symposium, in 1980, was again held in Houston. István Halász and Csaba Horváth received the Tswett Award. This was the first time that among the recipients one was the student of the other: Horváth received his Ph.D. at the University of Frankfurt, in 1963, carrying out his thesis work in Halász' laboratory. Fig. 23 stems from the award presentation. Both Halász and Horváth were born and grew up in Hungary and, together with Ervin Kováts and myself, were often jokingly called members of the Hungarian mafia in chromatography; Fig. 24 was made at this meeting. Fig. 25 is another shapshot made at the reception.

Our Sixteenth Symposium was held in 1981, in Barcelona, Spain, in the auditorium of the Autonomous University of Barcelona. The Tswett Chromatography Award was presented at the meeting to Arnoldo Liberti, professor at the

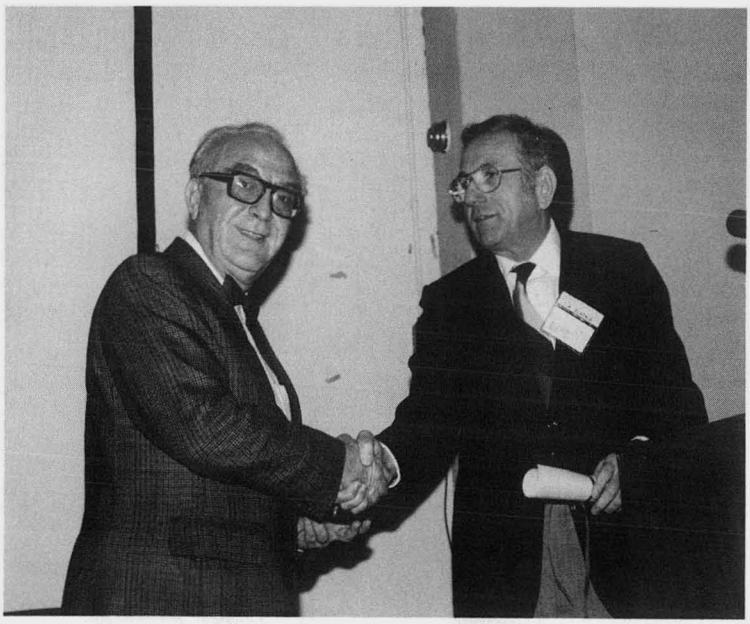

Fig. 23. 1980 Houston Symposium: István Halász (Saarbrücken University, F.R.G.) receiving the Tswett Award from Al Zlatkis.

Fig. 24. 1980 Houston Symposium. Right to left: Csaba Horváth (Yale University), E. sz. Kováts (EPFL), István Halász (Saarbrücken University) and L. S. Ettre.

University of Rome and head of the Italian Air Pollution Institute, and to Bob Sievers of the University of Colorado; both have made significant contributions to the use of gas chromatography in environmental analysis. The Barcelona meeting was also connected with a side trip for Al Zlatkis and myself. Originally, the Tswett Award was

Fig. 25. 1980 Houston Symposium. Right to left: Mrs. Zlatkis, J. K. Haken (University of New South Wales, Kensington, Australia) and R. E. Sievers (University of Colorado).

to be given to three persons, but Professor Sakodynskii of the Soviet Union could not participate at the meeting in Barcelona; thus, we decided to go to him. We combined this with an invitation by the Academy of Sciences of the Georgian Soviet Socialist Republic to lecture in Tbilisi and presented the award to Karl Ivanovich Sakodynskii there. Our lectures and the presentation made the first page of the September 26, 1981, issue of *Zarya Vostoka*, the official newspaper of the Communist Party of Georgia and of the Ministerial Council of the Georgian Soviet Socialist Republic. Fig. 26 shows us presenting the medal to Karl Ivanovich.

In 1982 we actually had two meetings. The Seventeenth Symposium was held again in Las Vegas; Arthur Karmen and Sandy Lipsky (Fig. 27) received their Tswett Award there. Seventy-four papers were presented at the meeting and one-third of these were part of a special session honoring the 65th birthday of Evan Horning. Since 1960 Evan, his students, collaborators and friends throughout the world established a new school, which has virtually revolutionized analytical biochemistry, by developing new methods and techniques and combining gas and liquid chromatography with other

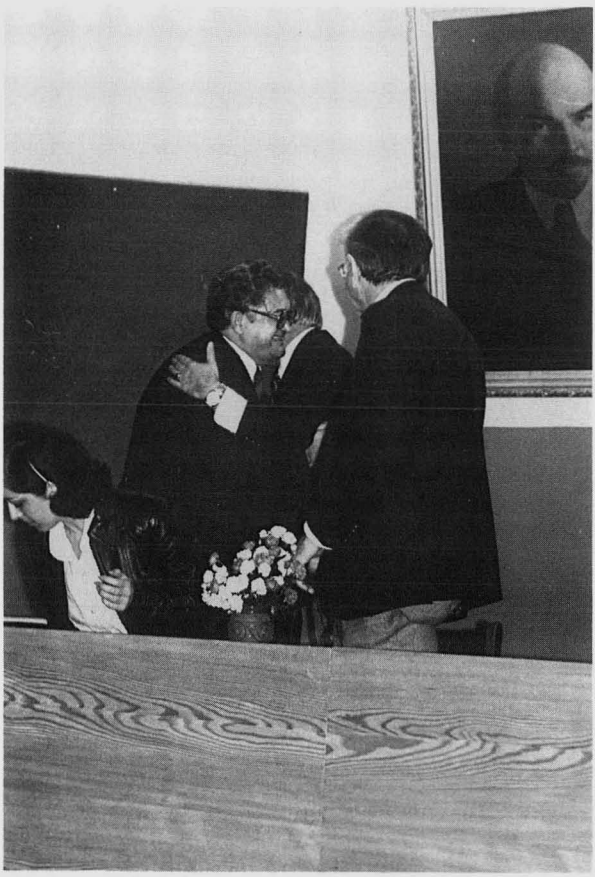

Fig. 26. Presenting the Tswett Award to K. I. Sakodynskii, in Tbilisi, in the auditorium of the Institute of Physical and Organic Chemistry, Academy of Sciences of the Georgian S.S.R.

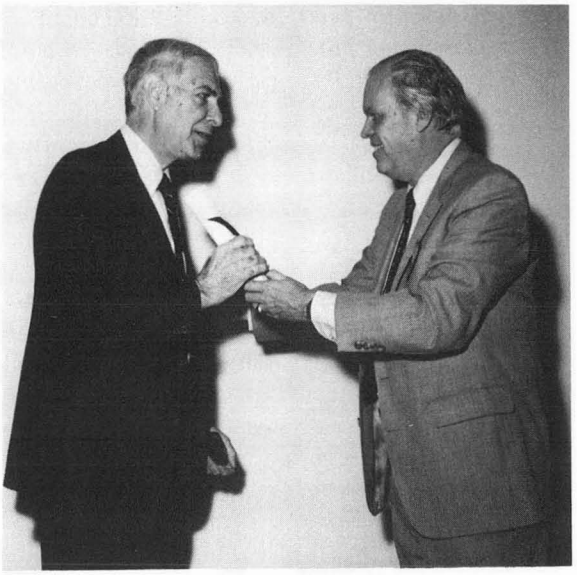

Fig. 27. 1982 Las Vegas Symposium: S. R. Lipsky (Yale University Medical School) receiving the Tswett Chromatography Award.

modern instrumental techniques. On this occasion, a special banquet was also held (Fig. 28).

From Las Vegas we flew directly to Tokyo and held our Eighteenth Symposium there. In the last decade, the Japanese have achieved the highest level of industrial

Fig. 28. At the banquet honoring the 65th birthday of Evan C. Horning (in middle). Right: Marjorie G. Horning.

development and production. In chromatography, Japanese scientists have excelled for many years, and we were happy to accept their invitation to organize a meeting there. Our meeting was cosponsored by a number of Japanese scientific societies, and they did an excellent job in its organization. The chairman of the local Organization Committee was Professor G. Muto, the president of the Japan Society for Analytical Chemistry. In an editorial published in the April 16, 1982, issue of *Asahi Evening News*, one of the two English-language daily newspapers of Tokyo, he expressed his hope that the Symposium "will also stimulate the young scientists in Japan to further and better their research".

A total of 102 papers were presented at the Symposium, 77 of them (75.5%) from Japan and 25 (24.5%) by scientists from 12 other countries, making the Symposium truly international. We honored two Japanese scientists with the Tswett Award: Hiroyuki Hatano of Kyoto University, the doyen of Japanese chromatographers, and Nobuo Ikekawa of the Tokyo Institute of Technology, who pioneered in many investigations of biologically important substances.

The opening session represented a special challenge to me, because I presented the usual opening remarks *in Japanese*; it took quite a time to learn the pronunciation.

Our Nineteenth Symposium —representing the 20-year anniversary of our first symposium— was held in 1983, in Amsterdam. This Jubilee Symposium was very well attended: almost 500 scientists participated at it, presenting more than 100 papers from which about 80% were included in the Proceedings Volume. In the Foreword, Al Zlatkis characterized the evolution of chromatography in the past twenty years in the following way:

> "When we started our symposia, most of the applications were still in the petrochemical and chemical industries. Since then, gas chromatography has become an indispensable technique in biochemical and clinical applications and in protecting our environment. Indeed, the significant improvements in cleaning up our environment would not have been possible without the availability of this, most sensitive technique.
>
> In 1963 liquid column chromatography was an empirical, laboratory method. (...) In the second part of the 1960s, researchers slowly started to apply the knowledge accumulated in the theory and practice of gas chromatography to this field. Around 1970, liquid chromatography suddenly started its exponential evolution catching up and even surpassing gas chromatography in many aspects. A similar evolution could be followed in thin-layer chromatography continuously improving the reliability, reproducibility and sensitivity of the technique.
>
> The evolution of chromatography would not have been possible without the continuous improvement in instrumentation. Here also, the past twenty years have brought significant changes; in fact, modern liquid chromatography could not have been possible without the development of sophisticated, high-pressure systems in use today."

At this symposium, we presented the Tswett Award to three scientists. Egil Jellum received it for his pioneering work in the utilization of combined chromatographic and mass spectrometric methods for the identification of metabolic diseases, and Gerhard Schomburg for his pioneering work in the development of multidimensional gas chromatography. In addition, it was felt that the time had come to present this award to the person who for twenty years was responsible for the organization of our symposia: Al Zlatkis. The citation of his award very properly referred to "his continuous contributions to the science of chromatography during the past quarter century, as a researcher and teacher".

The Twentieth Symposium was held in 1984, in New York City. The two

scientists receiving the Tswett Award not only represented a new generation of chromatographers, who, in 1963, had not even started their studies, but also represented a teacher–student association. The award was given to Milos Novotny of Indiana University, who, since 1968, significantly contributed to the field of high-resolution gas and liquid chromatography; and to Milton Lee of Brigham Young University, the first graduate student of Milos at Indiana University, for his research in high-resolution gas and supercritical-fluid chromatography.

In 1985, we travelled for the first time to Scandinavia, and had our Twenty-first Symposium in Oslo. The Tswett Award was given to John Knox of the University of Edinburgh, U.K. (Fig. 29) for his fundamental investigations on the theory and practice of modern liquid chromatography, to Karel Macek (Institute of Physiology, Prague, Czechoslovakia) for his pioneering activities in paper chromatography, and to Colin Poole (Wayne State University, Detroit, MI, U.S.A.), again a member of the new generation, for his achievements in the fields of selective detection, derivative formation and novel stationary phases in gas chromatography. After some years this was the first time that we again had participants from the U.S.S.R. and Fig. 30 is a group photo showing them with some friends.

In 1986 we again held two symposia. The Twenty-second Symposium was held in Houston, in September. We presented the Tswett Chromatography Award to Ernst Bayer for his many contributions to gas and liquid chromatography, to Carl Cramers of Eindhoven University of Technology, for his achievements in capillary gas chromatography and the combination of gas chromatography and mass spectrometry

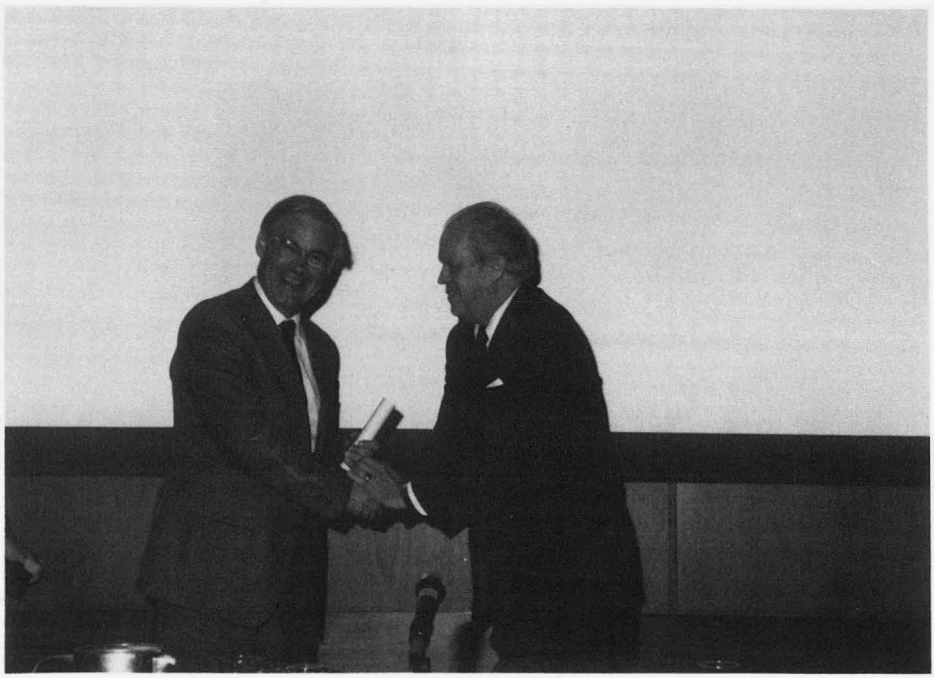

Fig. 29. 1985 Oslo Symposium: John H. Knox (Edinburgh University, U.K.) receiving the Tswett Chromatography Award.

Fig. 30. 1985 Oslo Symposium. Left to right: R. E. Kaiser (Institute for Chromatography, Bad Dürkheim, F.R.G.), Al Zlatkis, K. I. Sakodynskii (Karpov Institute, Moscow, U.S.S.R.), B. V. Ioffe (University of Leningrad, U.S.S.R.), Arthur Karmen (Albert Einstein College of Medicine), L. S. Ettre, Galina Malikin (Albert Einstein College of Medicine, a former student of Professor Ioffe).

Fig. 31. 1986 Houston Symposium. Left to right: R. E. Kaiser (Institute of Chromatography, Bad Dürkheim, F.R.G.), C. Cramers (Eindhoven University of Technology, The Netherlands) and F. Bruner (University of Urbino, Italy).

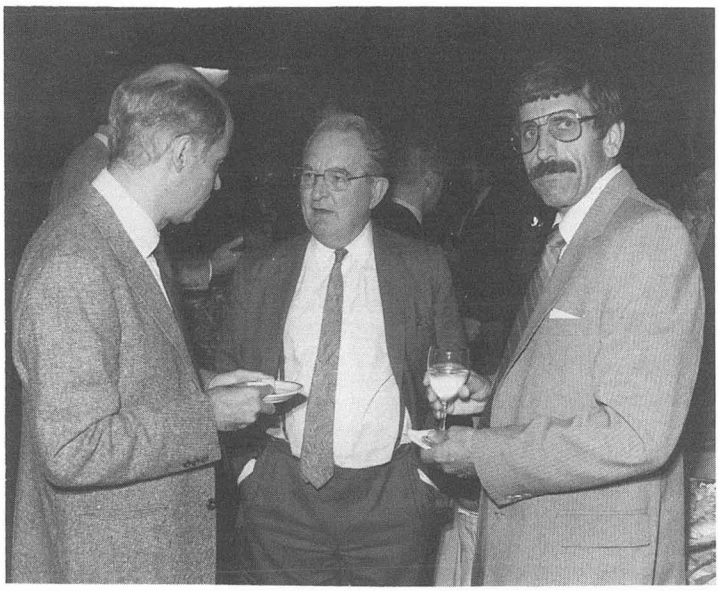

Fig. 32. 1986 Houston Symposium. Left to right: David Nurok (Indiana University–Purdue University at Indianapolis), D. H. Desty and Wolfgang Bertsch (University of Alabama).

and to Barry Karger of Northeastern University, for his pioneering work in modern high-performance liquid chromatography. Figs. 31 and 32 were taken during the reception.

A couple of weeks later, we were again in Japan, on the invitation of our friends. Our Twenty-third Symposium was held in Chiba, on the east side of Tokyo Bay. The Tswett Award was presented to Shoji Hara of Tokyo College of Pharmacy for his contributions to the field of thin-layer chromatography, and to Hiroshi Miyazaki of Nippon Kayaku Co., Tokyo, for his achievements in the study of biologically important substances and drug metabolism utilizing chromatography and isotachophoresis. One of the sessions of the symposium was convened as a tribute to Evan Horning on the occasion of his seventieth birthday; and a special evening session honored the memory of Sandy Lipsky, Professor Emeritus of Yale University, who passed away in the brief period between the meetings in Houston and Chiba. Originally, he planned to participate in these meetings and to present a paper on his latest research on high-temperature capillary gas chromatography; however, he was already very ill during the Houston Symposium (where I read his lecture) and passed away a few days later. With his death a true giant of chromatography left us, an active participant at every one of our symposia, and a dear friend.

We have one more brief stop in our journey. The Twenty-fourth Symposium was held in September 1987, in West Berlin. Three Tswett Chromatography Awards were presented there: to Marjorie Horning of Baylor College of Medicine, for her life achievements in the use of chromatography in therapeutic drug monitoring and the study of drug metabolism, to Daido Ishii of Nagoya University, for his pioneering work in the utilization of small-diameter columns in liquid chromatography, and to

Jan Sjövall of Karolinska Institute, in Stockholm, for the utilization of chromatography in biology, biochemistry and medicine. This Symposium provided the opportunity to the friends and former students of Professor Ernst Bayer to celebrate his sixtieth birthday with a special session.

With this, our journey in time is over: we arrived here, in Minneapolis at our Twenty-fifth Symposium. And with this, the job of your chronicler is finished.

THE TEACHING OF HISTORY

I am often asked: why are you so much interested in the past? Why should we waste our time on history? Aren't the techniques and methods used 25 or 30 years ago outmoded and irrelevant for the solution of our present problems? Why should we bother to read papers presented a long time ago? After all, we don't even have enough time to read what is published today.

Let me finish this historical journey by addressing this question. At the end of 1986, I had the honor to be invited to lecture at Technion, the Israeli University of Technology, in Haifa. There, visitors are treated to an audio-visual presentation about the history of the school and the evolution of technology in the past 50 years; and in it, the same question is also raised. It is answered by the following sentence: **The present is the future of the past, and the past of the future.** Everything we know now follows from the work which had been done in the past: we are its future. And what we do today will represent the past for the future generation. Chemistry of today could not exist without the pioneering work of our forefathers; and we wouldn't be here without Tswett's work, and the activities of the pioneers in the thirties and fourties. Thus, to know what has been done in the past 25 years and by whom, is not simply a matter of curiosity: knowing it, learning from it, can shorten your present work, just as our work was helped by the results of the generation before us. The situation will be the same 25 years from now: that generation will —and should— learn from your present-day work, and continue it.

EPILOGUE

The *International Symposia on Advances in Chromatography* originated 25 years ago by Al Zlatkis have been an important forum, where chromatographers have reported their results, and the proceedings of the twenty-five meetings are and will remain an important source of knowledge for future generations. Studying these volumes, the evolution of gas and later modern, high-performance liquid chromatography can be followed. On the occasion of this anniversary, I would like to thank all of my colleagues who, in these 25 years participated in our meetings and contributed to the advancement of chromatography. And, last but not least, I would like to thank Al Zlatkis for organizing these meetings.

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CHROMATOGRAPHY AND THE DISCOVERY PROCESS

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SUMMARY

Though each science has its own special features, chromatography neatly and simply illustrates most of the significant characteristics of the discovery process. These include multiple discovery (and its logical counterpart "adumbrationism"), missed discoveries, the dominance of a problem, the crucial role of observation, the advantages of tangential approach, analogy, and serendipity. The story of chromatography also brings out the contributions of the craft and scholarly traditions, the influence of human interactions, and the impact of the intellectual climate. The paper gives examples of these various factors, and stressed the importance to science of collecting information now on how discoveries are actually made, particularly for those discoveries which may not seem to be paradigmatic.

From the mists of chromatographic time, two strong figures emerge, the later more clearly than the earlier.

Michael Tswett (1872–1919) (his surname is the Russian word for colour, especially the colour of plants) died largely unrecognised in Voronezh. Opinions vary as to the essence of his discovery, although his work was fundamental for chlorophyll chemistry and he gave a very clear account of the processes we now understand as chromatography. There were many [such as Schoenbein (1861), Goppelsröder (1861 and later), Day (1897, although he appears to have been overrated in a good deal of the literature), Engler and Albrecht (1901)], who had published related work of which Tswett appears to have been well aware. Indeed Tswett makes mention of Goppelsröder in his classic paper[1]. (For a particularly clear and detailed account of the early history of chromatography, see ref. 2). Further, Bayer[3] has shown that the basic principles of gas chromatography were put forward and demonstrated practically as early as 1512; Tswett is probably remembered chiefly because his real interest lay in plant pigments, and it was Edgar Lederer's work on carotenes in the 1930s that effectively resuscitated his method. Previously it had been condemned by Willstätter and Stohl (1913) because they had been unsuccessful in the preparative purification of chlorophylls using "Tswett columns"; apparently they had failed to notice what Tswett had already made clear, namely that chlorophylls are destroyed by "aggressive" adsorbents and therefore required materials such as powdered sucrose or inuline (see ref. 4). Finally one must recognise that Tswett's proposals were against the current of the time, where synthesis, isolation and purification were the philosophical essentials of organic chemistry, while Willstätter, with his traditional techniques, was

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the great figure in the subject. It is perhaps worthy of comment that organic chemists today are still relatively uninterested in isolating all the components either of natural material or of experimental reactions.

Archer Martin (born in 1910) (whose name in English refers to a clever and remarkably consistent bird –there were twelve pairs of martins at Selbourne two hundred years ago in the time of Gilbert White and I am reliably informed by my ornithological colleagues that there are twelve pairs in the parish today) invented partition chromatography with Synge in 1941[5], paper chromatography with Consden and Gordon in 1944[6], and gas–liquid chromatography (which had been "predicted" in the 1941 partition paper) with James in 1951[7] (and which was then rapidly taken up and developed by ICI, BP and Shell among others). As is common in the history of discovery there were others who were working on related lines, and who would probably have got there in time if Martin had not got there first. Hesse, Cremer, Claesson, Glueckauf and even myself were all working on gas chromatography before Martin, but we did not see the essential step (to use partition rather than adsorption columns). And then there is Bayer's chap. But we must avoid the pitfalls of precursoritism or what Merton[8] has called "adumbrationism", the attempt to discover some earlier historic event which might have changed the development of science, but for some reason or other did no so. It is all too easy to see depth in mere darkness.

Martin has always claimed that his success with partition chromatography arose in part because he and Synge were faced with a real problem, the separation of amino acids, while the crucial step from a clumsy counter-current apparatus was the realisation that it was not essential to move *both* liquids. He and James shifted to gas chromatography after some unsuccessful attempts to automate fractional crystallisation largely, so we are told, because Martin was certain it would work and something surefire might be needed to boost James's morale. I recall once, in the early days, being shown round an industrial plant with Martin. Our hosts were cock-a-hoop about a gas chromatographic analysis that they had taken many months to develop. They would show it to Martin on condition that he kept it a secret. Keeping scientific secrets is not Martins's style, so he suggested that he would at once outline how he would have done the job, and that if their system was essentially different then he would prefer not to see it. It was not.

One curious feature that has distinguished chromatography from other scientific techniques is that it has not for the most part depended upon previous technical developments in other fields, as for example the wartime developments in radar without which modern NMR would have been impossible and radioastronomy would have been hamstrung. (On the other hand, there is little doubt that it benefitted from a change in scientific climate or philosophy). A possible exception is the construction of high-sensitivity detectors, the need for which was clearly recognised by Martin ("I want something much more sensitive; we shall need detection of fractions of a microgram"), and which were crucial to high-performance gas chromatography. But the history of chromatography has been replete with multiple or independent discoveries. I will mention two that are personally well known to me. Several people had thought of developing capillary columns, but the most significant step was taken by Marcel Golay. He was, as I was, a consultant to Perkin-Elmer, and had been asked to turn his mind from the exotica of communication theory to the practicalities of gas chromatography. The latter as it then existed was however too much of a craft, and he demand-

ed that he should work on the theoretically simpler system of an open rather than a packed tube. He then developed a theory of chromatography entirely from scratch. He showed it to me. The first half was quite familiar and had already been independently published by others; the second was essentially new. Perhaps there is a moral here about the dangers of overindulgence in the literature, although my own experience in chromatography would suggest that the reverse is the more common error: I have more than once encountered the view that something had not been done because it was not published in *Analytical Chemistry*. Golay also found it hard to realise that his open-tubular columns could have any application. I thought I was the first one to convince him that they did, but he still went on to drop a clanger about their impracticability at Amsterdam. This is confirmed by Dijkstra[9] who had also been working on capillary columns, but who had failed to do the calculations that Golay had done and was therefore working with gas flowrates that were much too large for high efficiency. My second example is concerned with temperature programming. I was the first to publish anything on this technique[10]. I was not particularly proud of the concept, for it seemed to me to be the obvious gaseous analogy of gradient-elution liquid chromatography which had recently been invented by Williams and others. I was even stupid enough not to consider patenting the idea, and so avoided the perils of becoming a millionaire. However, my real point is that temperature-programmed chromatography was then independently invented by at least three other groups, the chief investigator of the last being awarded a medal for his discovery.

I have already mentioned examples of *forgotten* discoveries in the history of chromatography, including chromatography itself and gas–liquid chromatography. We must suspect that there are many others and someone not driven foreward inexorably by the brilliance of his own thinking might well find it profitable to peruse some of the older literature for clever ideas that have never been properly developed. My own favourite here is the paper on "Electron attachment spectroscopy" by Lovelock *et al*[11], which as I understand was never followed up because of difficulties with technical assistance in Houston, which outsiders could never believe. I have also over the years been impressed by the bandwagon effect in chromatography, which tends to carry most practioners along whatever road is currently in fashion. I would suggest in particular that the non-analytical aspects of chromatography have been somewhat neglected, while the reproducibility and taxonomy of the subject has not yet been developed so as to make chromatography the standard physico-chemical tool it might be.

I believe it was Mark Twain who pointed out that while everyone would talk about the weather, nobody actually seemed to be doing anything about it. To some extent it is the same with scientific discovery. Martin began his Nobel lecture in 1951 with these words: "If enough histories, written while the ideas are still fresh in the minds of the peoples concerned, are available for a variety of discoveries of inventions, it may eventually be possible to lay down some of the principles required to facilitate the obtaining of fruitful results in scientific research in general. Clearly also the background of knowledge at the time the advance was made will be best understood if the history is as recent as possible." This will be the main theme of this paper.

How then are discoveries made? I believe, contrary to what most philosophers seem to have supposed, that there is no one royal road: there are many different ways of making discoveries but certain circumstances reappear with sufficient regularity to

suggest that they might be useful as guides. The first perhaps is the need to solve a *problem*. We cannot imagine that Archimedes was the first to cause his bath to overflow. When Pavlov was asked by his students how they might become as inventive as he was, his reply is quoted as "Get up in the morning with your problem before you. Breakfast with it. Go to the laboratory with it. Eat your lunch with it. Keep it before you after dinner. Go to bed with it on your mind. Dream about it." In a somewhat different context Lenin is said to have made similar remarks about a revolution. Tswett and Martin were both brought to chromatography to solve specific problems, and this seems to have been the case with the vast majority of the classic chromatographers. In fact they were not prone to call themselves chromatographers or even analytical chemists but biochemists, chemical engineers or medical researchers.

Cross-fertilisation seems also to play a frequent role in scientific discoveries. Many if not most of the chromatographic advances have been made by those who have come into the field from outside. Furthermore, the rapid advance of chromatographic methods has been enhanced by the readiness with which ideas were exchanged. Martin himself set the tradition (see for example the remarks of Scott[12]) but the banner was readily taken up by the Chromatography Discussion Group under Desty and of course by the series of conferences organised by Zlatkis of which this is the Jubilee.

Analogy can often be useful. I have referred above to the analogy of partition chromatography and counter-current processes, and of temperature programming and gradient elution. High-performance liquid chromatography seems to have been created at least in part by out-of-work gas chromatographers, who wondered why they should not be able to get similar high efficiencies with flowing solvents as with flowing gases. But analogy is not always right, and the early years of gas chromatography were plagued by low pressures at column outlets because this is what one did in distillation.

Simplicity has its philosophical counterpart in Occam's razor, but is beautifully exemplified in chromatography where the crucial ideas can be explained without complexity and even to the layman. *Relaxation* or turning aside from the immediate problem has often been scientifically productive. Kekulé is said to have mused about chemical structures in front of the fire and on the top of the Clapham omnibus. Heisenberg arrived at the essentials of his quantum matrix mechanics while he was escaping from hay fever by the sea at Heligoland. There must be parallel examples in chromatography, but I have not been made aware of them. However, little seems to have been achieved without *hard work*, despite the simplicity which eventually results. Many of us have had the experience of thinking up but not thoroughly developing an idea, which is then later done properly by others. For me pheromones is a case in point.

Observation is crucial. I have had more or less the same experience with all new research students and over some forty years. They expect to get a certain result; usually I have to admit a result that I have suggested. When they do not get it, their first reaction seems to be to do the experiment again. Of course they should check to see whether they have made some simple mistake, but they should also consider (and this seems to strike most of them as quite bizarre) that they may have observed something essentially new. I once had a pupil who was an unusually inept experimentalist. He was studying the catalytic polymerisation of olefins, but could never

inject the same amount from one experiment to the next. To overcome this difficulty I proposed that he worked with a standard mixture of olefin and an inert paraffin marker. He soon had the paraffin polymerising as well, and would not take no from me as his answer. His insistence led us to investigate further and we were thus able to uncover some slow diffusional processes. Later I published a note on this, and was very interested to find that a number of other research supervisors had been presented with similar observations, but by less determined students. Or as Hesse quotes Professor Meerwein about a colleague: "The poor man is too educated. As soon as he has an idea, he immediately knows why it should not work, and therefore he never tries anything"[13]. Observation however needs to be *careful* observation. Many years ago we were measuring some thermodynamic effects by gas chromatography. This required precise control of column temperature which we set out to achieve with vapour baths. Stupidly I forgot that vapour purity was not enough so that the final calculations provided a greater scatter than we had hoped for. Then the penny dropped, but fortunately my student had indeed been very careful and had noted the exact times of all his measurements. We were thus able to correct for the atmospheric variations in pressure by reference to the Geography Department of the University which has kept detailed records in Oxford over many years.

Then there is *serendipity*, a feature which I have found stressed by nearly all scientists that I have spoken to about their discoveries. Martin claimed that it was the accidental presence of 1% ethanol as stabiliser in his chloroform solvent that prevented the amino acids remaining at the top of his column[14]. The argon-ionisation detector, so Lovelock tells us, was discovered because the stores had temporarily run out of cylinders of nitrogen[15]. Porath's work on size-exclusion chromatography began with some electrophoretic experiments in which the current by neglect had not been switched on[16]. Hollis, as I recall, developed porous polymers as stationary phases for gas chromatography because he chose by accident to investigate first the only suitable polymer from a range which he had been sent in the hope that he could find something for which they might be useful. Ettre[17] claims that his entry into chromatography was a result of his being falsely identified as an analytical chemist. Giddings[18] traces his theoretical contributions to chromatography to the curious chance of his being instructed to go into chromatography by Henri Eyring at a time when he had just been taking a graduate class on the "Principles of physical statistics". The technique of stopped-flow chromatography[19] turned out to be much better than I had expected, because I had not fully thought out the functions of the chromatographic column: at times it can be fortunate that it is so easy to do experiments in chromatography.

On *fraud* and *error* it is perhaps wise to pass, but I suspect most of us have found it difficult to repeat exactly the work of others.

Rejection seems to be the natural fate of many good new ideas. We think perhaps of Tswett, but most of us were discouraged in our early efforts at chromatography. Gerhard Hesse[13] relates that the disbelief in chromatography in Munich in 1930 was so well established that he was angrily instructed by his supervisor Wieland to "stop this stupid thing". I too was very pointedly told that I was wasting my time, until I was rescued by a visit from some chemists in ICI, and I understand that Howard Purnell was thought by Professor Norrish to be going down a mere bypath[20]. Evan Horning was strongly opposed by scientists and scientist administrators

in his desire to introduce the new ideas and new techniques of chromatography into medical research[21]. When Keene Dimick started the Aerograph company (through which he was later to become of course a millionaire) it was treated as something of a joke by his colleagues in government service who were convinced he would soon be back with them again[22]. In response to Michael Lederer's proposal to Elsevier that there should be a journal for chromatography, the director was far from enthusiastic and wondered "would this chromatography last?"[23]. The referee of the paper by Zlatkis and Lovelock which combined capillary chromatography with a sensitive ionisation detector was anxious to reject it as the chromatograms were too good to be real[15]. I was told that he suspected they had been drawn with a ruler.

Many discoveries (like Columbus) arise because someone looks where no one has looked before or, in science particularly, because of the advent of a new tool such as the telescope, the microscope or microwave technology. For Tswett (quoting Descartes) "every scientific advance is an advance in method". Jack Kirkland[24] traces his enthusiasm for chromatography to the fact that Dal Nogare was able in three hours to solve a problem which had been bugging him for many weeks. "Sandy" Lipsky[25] recorded his own conversion when he read an article by Martin in the *Biochemical Journal* which described the separation of the fatty acids, a problem with which he had long been wrestling. I remember that many years ago we showed[26] how it was possible to identify a whole string of volatile silanes (the silicon analogues of the alkanes) by gas chromatography. The work rippled few Anglo-Saxon waters, but one of my research students found later that he had saved the reputation of a young continental colleague who had claimed to have made iso-silobutane, which the "great Professor Stock had failed to make many years before".

Now I have tried to suggest that it is important to know the way in which discoveries are actually made. There is indeed something of an anthropology of science which will be lost if no attempt is made to preserve it. This means therefore that some record should be kept of the discovery process as well as the discovery itself, although this flies in the face of all the recent traditions of the scientific literature. In particular the editors of scientific journals, no doubt correctly worried by their problems of space, strongly discourage such a procedure: they seem trained to prefer the supposedly rational to the real. Philosophers were long eager to explain how science was done, but more recently they seem to have given up the ghost of even telling us how it should be done: they concern themselves more and more with only the logic of testing and proof. Thus it is only scientists themselves who can really tell us what is done. But why in particular chromatographers? Why you?

To my mind there are at least six good reasons. Firstly and most obviously, chromatography has been my own specialist interest and clearly among the interests of you my audience. Secondly, while I have always found that chemistry is a glorious conversation stopper, chromatography and what chromatography does is something that one can, without too much difficulty, explain to the traditional man or woman in the street. I believe it should play a much larger role in chemical education, a point I have laboured elsewhere[27]. Moreover, thirdly, our subject lies at the very heart of chemistry, a theoretically unreal realm since it is concerned with the properties of pure substances, which like vacua and for essentially the same reason are abhored by nature. I remember Keulemans pointing out to me that the Dutch have it right when they call chemistry "scheikunde" (the art of separation). Fourthly, as I have already

indicated, the traditions of chromatography involve the ready, frank and honest exchange of ideas. Fifthly, it is a subject that is still very alive and vigorous. The problem so often with the history of science, and especially for scientists, is that it is difficult for us to recast our minds into the ways of thinking of a past age. Who could now resurrect the full awe of the Pythagoreans who seem to have been the first to recognise that numbers play a role in natural phenomena or the theological restraints that bedevilled Newton and Darwin? When Richard Trevithick first contemplated using a steam engine as a locomotive, he had seriously to consider whether it really would be possible to use a wheel to provide traction rather than merely roll along behind traction. Apparently he only became convinced after he and a friend had removed the horse from a cart and demonstrated to themselves that it could be moved uphill by turning the spokes by hand[28]. Also of course we no longer have access to all the relevant facts: they are conveniently but misleadingly replaced by imagination. Sixthly, I would wish to suggest that for most of us the more mundane discoveries are actually more significant than the Kuhnian paradigms: they are so much more like the discoveries that scientists actually make. It may even be that the Newtons, the Einsteins and the Martins are a race apart with their own peculiar roles: though I have to admit that I doubt it. Moreover one of the problems with famous discoveries is that they have been too often enquired into; they tend to generate myths. Mendel and Tswett are probably examples. Certainly it has been shown that Fleming could never have discovered penicillin in the way he later described[29].

The study of chromatography also raises the intriguing question whether there may be other simple techniques which we are missing, and which later generations will regard as curiously obvious. Or is the whole of scientific research a little like oil: something of a wasting asset from which we have been particularly fortunate to benefit?

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CHROMSYMP. 1541

ADVANCES IN THE USE OF COMPUTERIZED GAS CHROMATOGRAPHY–MASS SPECTROMETRY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH RAPID SCANNING DETECTION FOR CLINICAL DIAGNOSIS

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SUMMARY

A multi-component analytical system designed for the diagnosis of metabolic disorders is described. The urinary components are separated by a variety of chromatographic techniques, including automated amino acid analysis, high-performance liquid chromatography with diode-array detection and gas chromatography–mass spectrometry with a computerized mass spectral library search for identification of organic acids. The complete system can be used to diagnose over 100 different metabolic diseases. The usefulness of the chromatographic system is exemplified by the pre- and postnatal diagnosis of glutaric aciduria type I, the diagnosis of lysinuric protein intolerance and of alkaptonuria. Drugs and diet may cause interfering metabolites, as exemplified by glycofurol, used as a solvent for intravenous drugs, and saccharin. It is predicted that chromatography and mass spectrometry will continue to be important diagnostic tools for many years ahead.

INTRODUCTION

Nearly 25 years ago, we began to use gas chromatography (GC) to study the metabolic disorder Refsum's disease (phytanic acid storage disease). The potential of using GC, particularly in combination with mass spectrometry (MS), to diagnose different metabolic diseases was soon realized. In 1969, the first GC–MS instrument was installed in our Institute at the National University Hospital of Norway. Prior to this, we had on several occasions used the GC–MS equipment at the Karolinska Institute in Stockholm, Sweden, *e.g.*, in 1967 to diagnose the first case of methylmalonic acidaemia[1]. GC analysis, including various extraction steps, hydrolysis and different methods of derivatization and separation on various packed GC columns, was put into systematic operation in 1970[2]. The identification of metabolites present in increased amounts in urine and serum because of an enzyme deficiency was carried out by mass spectrometry, followed by an off-line computer search against a mass spectral library of reference spectra, which we have programmed[2]. This GC–MS–computer system made it possible to detect and identify many unknown compounds within a short time, and proved to be well suited to the diagnosis and studies of a number of

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different metabolic diseases. Both we[3,4] and others[5–9] soon discovered several new diseases with the aid of GC–MS.

Our analytical system for detecting metabolic diseases has undergone considerable changes over the years, *e.g.*, to include the latest advances in chromatography and electrophoresis. In this paper, our current routine system used for diagnosis is described, followed by some recent applications.

EXPERIMENTAL

Patient selection and sample preparation

The patients selected for multi-component analyses usually have one or more of the following clinical "warning signals": progressive disease, recurrent disease, similar cases in the family, failure to thrive, metabolic acidosis, peculiar smells of the body and urine, recurrent vomiting, liver pathology, sopor/coma, convulsions and other neurological symptoms of unknown etiology. In addition to the clinical information, we require that every sample submitted for analyses be accompanied by information about drug intake. Interfering drug metabolites are a serious problem, which over the years has led to much loss of time and resources.

Usually 5 ml of urine (depending on the creatinine concentration) is acidified and extracted with diethyl ether and the organic acids are subsequently converted into the corresponding methyl esters with diazomethane. Other laboratories prefer to use, *e.g.*, ion exchange for the isolation of the acids and silylation to make them volatile before GC–MS analysis[6,7].

Sample treatment prior to automatic amino acid analysis includes addition of sulphosalicylic acid to remove small amounts of urinary proteins. HPLC analysis is carried out directly on urine (diluted about 10-fold, depending on the creatinine concentration). When pathological HPLC isograms and chromatograms are seen, sample clean-up with Bond-Elut cartridges (Analytichem, Harbor City, CA, U.S.A.) is carried out before HPLC analysis[10].

GC–MS analyses

Two GC–MS instruments are in daily use with a third as back-up. The first is a Hewlett-Packard (Avondale, PA, U.S.A.) 5970 mass-selective detector coupled to a gas chromatograph (HP 5890 GC) with an automatic sample injection system (HP 7673A 100) and an HP 300 data system. The second instrument is a Finnigan (Sunnyvale, CA, U.S.A.) Model 4021 C GC–MS system with an Incos/Nova 4 data system. The back-up instrument is a Varian-Mat (Bremen, F.R.G.) 112, which is operated when required and used manually. The GC–MS instruments are fitted with fused-silica capillary columns (30 m) and the coatings are usually SP-1000 or SPB-5 (Supelco, Bellefonte, PA, U.S.A.). Nearly all GC peaks are automatically identified by a mass spectral library search. The libraries now in use contain the mass spectra of about 40 000 different compounds, including many drug metabolites and metabolites of biological interest, and the collection of 230 mass spectra of urinary organic acids published by Spiteller and Spiteller[11]. In addition to the computer search routines supplied commercially (Finnigan and Hewlett-Packard), we have developed a simple program which automatically identifies about 200 organic acids known to carry specific, diagnostic information.

HPLC with rapid scanning detection

The HPLC instrument is an LDC liquid chromatograph with two ConstaMetric pumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The diode-array detector is a Model 2140 rapid spectral detector (LKB, Bromma, Sweden) with an IBM-XT personal computer. The LC-18 reversed-phase column (Supelcosil) was obtained from Supelco, the Asahipak GS 320 H porous polymer column from Gasukuro Kogyo (Tokyo, Japan) and the Bond-Elut strong cation exchanger from Analytichem. An Aminex HPX-87H ion-exchange column (Bio-Rad Labs., Richmond, CA, U.S.A.) is used for the HPLC of lactate and pyruvate in cerebrospinal fluid in cases of suspected cerebral lactic acidosis[12].

RESULTS AND DISCUSSION

The complete multi-component analytical system-currently used in our laboratory is summarized below (for more details, see ref. 10). Before embarking on analyses, information on clinical condition and drug intake is an essential requirement.

Investigations begin with simple dip-stick tests for, *e.g.*, glucose, blood ketone bodies and reducing substances. Creatinine concentration is determined. Thin-layer and paper chromatography are used for the detection of mucopolysaccharides and carbohydrates, respectively. Quantitative amino acid analysis is performed with automated ion-exchange chromatography and ninhydrin detection. Organic acids are identified by computerized GC–MS with automated sample injection and an automated mass spectral library search. HPLC diode-array with a computer determines purines, pyrimidines, orotic acid, carbamylphosphate, succinylpyrines and other non-volatile compounds. Lactate/pyruvate in cerebrospinal fluid is also measured. Finally, assay of the suspected defective enzyme is carried out in leukocytes, biopsies, fibroblasts or amniotic fluid cells when required.

The complexity of this system, despite considerable automation, makes it impossible to carry out a large number on analyses on samples from patients. Fortunately, this is not required, as metabolic diseases are rare. Our current capacity is around 1000 complete patient evaluations per year.

Urine and blood samples from cases suspected of having metabolic disease are sent to our laboratory from hospitals in Norway. In many instances, the outcome of the analyses results in a correct diagnosis. Follow-up studies, *e.g.*, monitoring the effect of treatment and, in a few instances, prenatal diagnosis, are also carried out by chromatographic methods in our laboratory. Below we report some typical recent results, which illustrate how the various chromatographic techniques play important roles in diagnosis.

Glutaric aciduria type I

A 2-year-old girl had involuntary movements, epilepsy and hydrocephalus, and had been shunted when she was 1 year old. The urine analyses showed a normal amino acid chromatogram, but the organic acid profile, as determined by GC–MS, showed the presence of large amounts of glutaric acid and also some 3-hydroxyglutarate (Fig. 1). These diagnostic metabolites were recognized by the computerized mass spectral library search. It was clear that the patient suffered from glutaric aciduria type I, which is due to an enzyme defect (glutaryl-CoA dehydrogenase) in the degradation of the

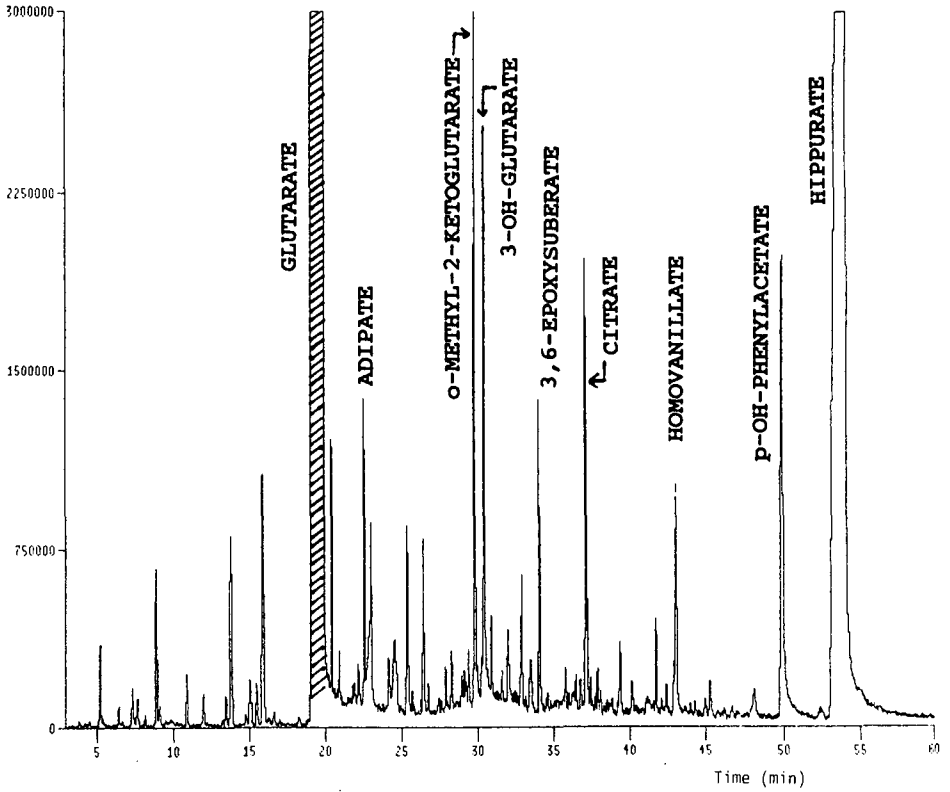

Fig. 1. Organic acid profile (total ion current *vs.* time) of urine from a 2-year-old girl suffering from glutaric aciduria type I. An SP-1000 fused-silica capillary column (30 m) was programmed from 80 to 220°C at 4°C/min, in a Hewlett-Packard 5970 GC–MS system. Note large amounts of glutarate and smaller amounts of the secondary metabolite 3-hydroxyglutarate.

amino acids lysine and tryptophan[13]. The patient is now given a diet low in these amino acids and low in proteins. The excretion of glutaric acid is frequently checked by quantitative GC–MS, using 2-methylglutarate as internal standard.

The following example illustrates the use of GC–MS in the prenatal diagnosis of glutaric aciduria type I after the discovery of two cases in the same family: the first child is a healthy boy; the second child (boy) was admitted to our hospital at the age of 2.5 years with symptoms similar to those of the patient mentioned above. Glutaric aciduria type I was diagnosed. Unfortunately, he died shortly afterwards. When a third boy was born, prenatal diagnosis had not been carried out, but his urine was analysed during his first days of life. Glutaric aciduria I was also detected in this child. Dietary treatment was immediately installed, and now at the age of 4.5 years his development is satisfactory. During the fourth pregnancy, amniotic fluid was obtained in the 16th week. After removal of the cells for culturing, the supernatant fluid was analysed by GC–MS in the selected-ion monitoring mode (the fragment of m/z 129 was used). It was evident that the glutaric acid content was not elevated compared with control amniotic fluid (Fig. 2). If the foetus had been afflicted, the glutaric acid

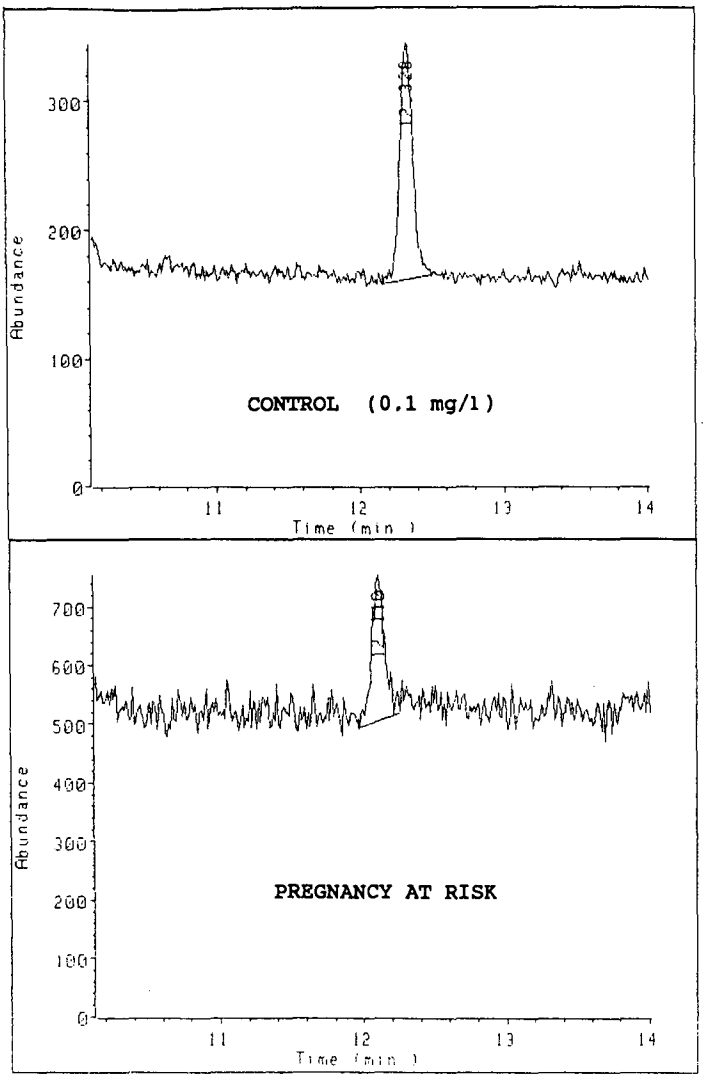

Fig. 2. Determination of glutaric acid in amniotic fluid from a control and from a pregnancy at risk by selected-ion monitoring. Dimethyl glutarate was monitored at $m/z = 129$. 2-Methylglutaric acid was used as an internal standard (not shown). The control sample contained 0.1 mg/l of glutaric acid in amniotic fluid, the pregnancy at risk sample even less, indicating that the foetus was not afflicted with glutaric aciduria type I.

concentration would have been about 50 times higher than that actually found[14]. This information could be passed on to the mother a few hours after amniocentesis, whereas the result of the enzyme studies became available only 3 weeks later and confirmed normal enzyme activity. Although GC–MS technology is very rapid and useful for the prenatal diagnosis of glutaric aciduria type I and 10–20 other diseases, *e.g.*, tyrosinaemia (determination of succinylacetone in the amniotic fluid) and isovaleric

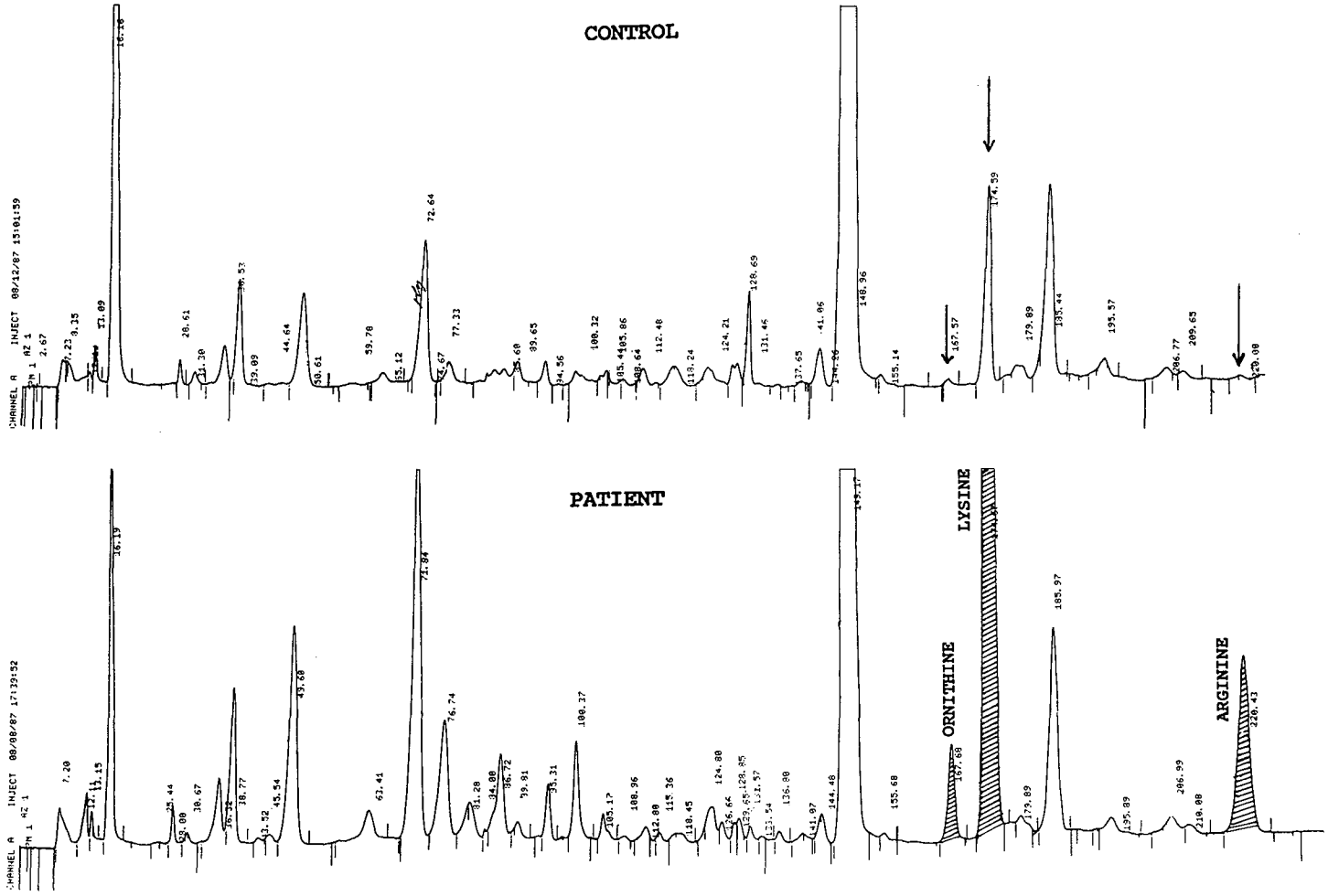

Fig. 3. Amino acid chromatograms of urine from a control (top) and from a patient (bottom) with lysinuric protein intolerance. Note the increased excretion of lysine, arginine and ornitine in the sample from the patient.

acidaemia (determination of isovalerylglycine in the amniotic fluid), it should be emphasized that, as a rule, enzyme studies and/or analyses at the DNA level are by far the most widely used techniques for the prenatal diagnosis of metabolic diseases.

Lysinuric protein intolerance

A 10-year-old girl had suffered for several years from frequent vomiting, particularly after intake of protein-rich food. There was a marked growth retardation. A urine sample was analysed with our standard chromatographic system. The organic acid pattern was normal, but the amino acid chromatogram (Fig. 3) and HPLC profile (Fig. 4) were pathological. The lysine excretion, in particular, was greatly increased, and increased amounts of arginine and ornithine were also found (Fig. 3). The HPLC trace, recorded at 278 nm (Fig. 4), showed the presence of large amounts of a compound that eluted at the same position as orotic acid. The absorption spectrum of this peak (Fig. 4, inset) as recorded by the diode-array detector was also identical with that of authentic orotic acid. Increased urinary excretion of lysine, arginine, ornithine (but not cystine) and orotic acid is characteristic of lysinuric protein intolerance (LPI)[15]. This amino acid transport disorder is very rare in most countries (the present case was the first in Norway), except in Finland, where more than 100 cases have been reported.

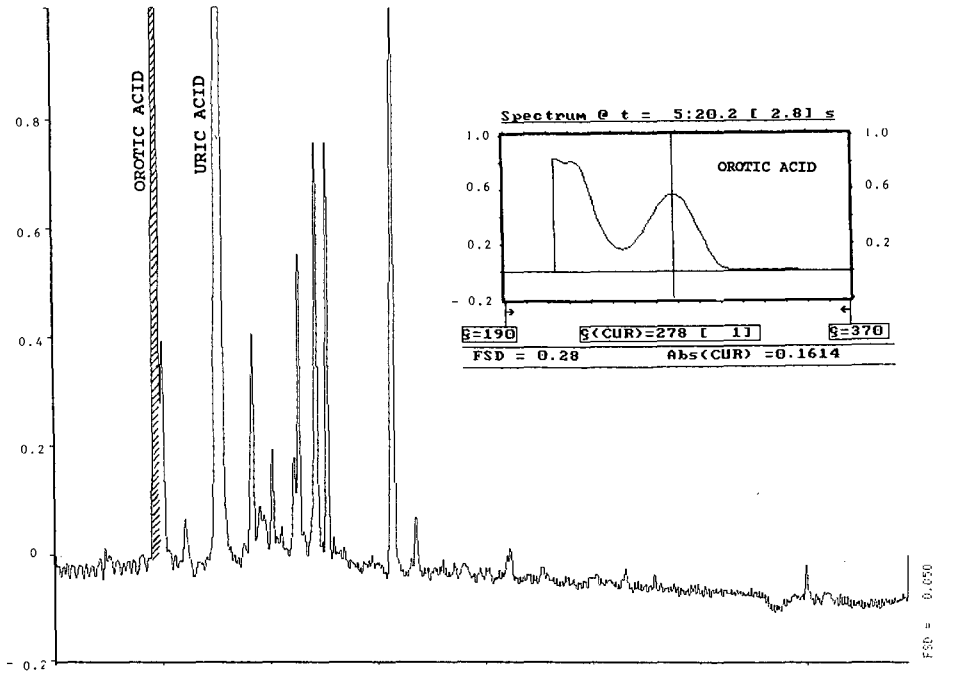

Fig. 4. HPLC profile of urine from a patient with lysinuric protein intolerance. The Supelcosil LC-18 column was eluted with a gradient system: starting eluent, 5 mmol/l H_2SO_4; end solution, 40% (v/v) aqueous acetonitrile; flow-rate, 1.0 ml/min; detection, 278 nm. The peak designated as orotic acid had a retention time and absorption spectrum identical with those of authentic orotic acid (inset).

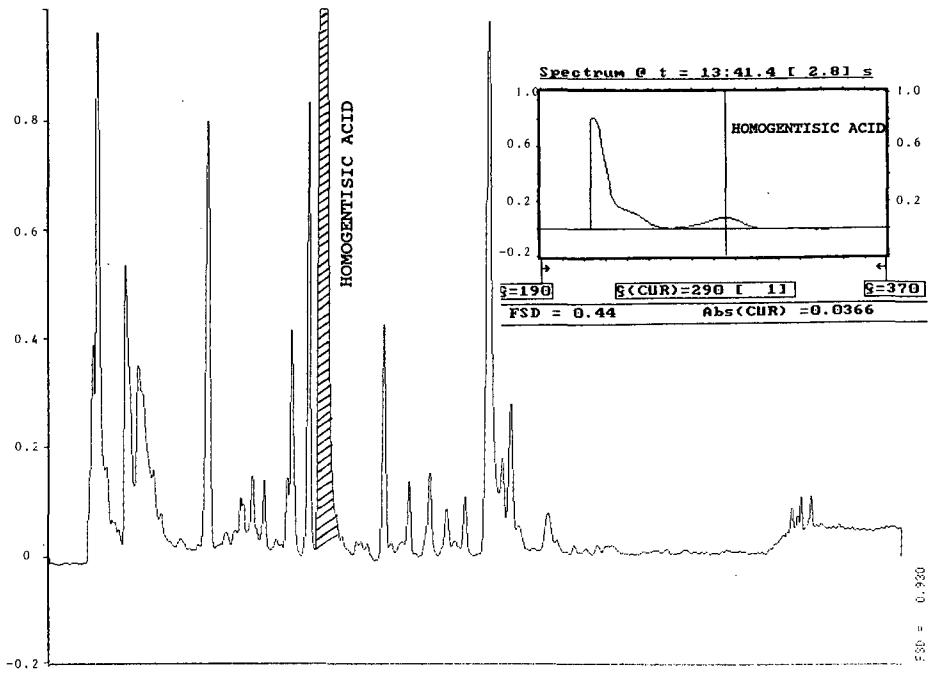

Fig. 5. HPLC profile of urine from a patient with alkaptonuria. Experimental conditions as in Fig. 4.

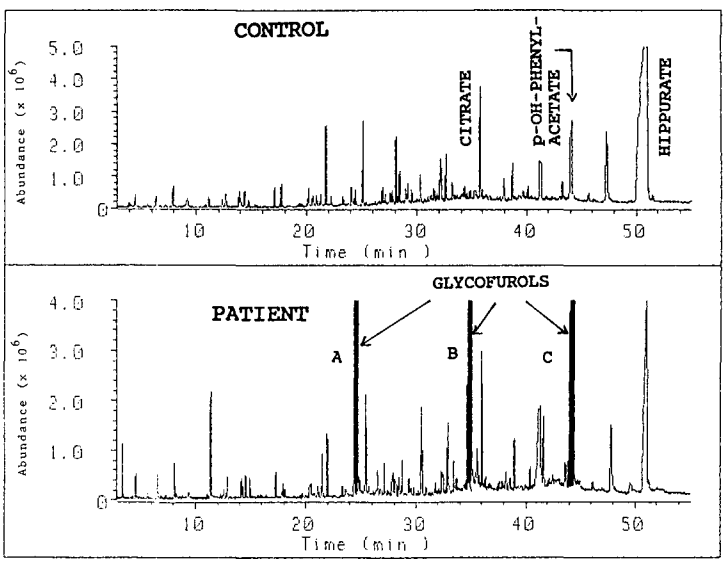

Fig. 6. Organic acid profile of urine from a control and from a patient who had received diazepam intravenously. Experimental conditions as described in the text and in Fig. 1. Peaks A, B and C are due to metabolites of glycofurol, used as a solvent in intravenous pharmaceutical preparations.

Alkaptonuria

Alkaptonuria (homogentisic acid oxidase deficiency) is one of the first inborn errors of metabolism ever recognized[16]. Children and young adults usually have no symptoms, but with age pigmentation of the sclera, cartillage or fibrous tissue occurs. Later, the patients develop arthritis. Because of the enzyme defect, homogentisic acid accumulates and is excreted in gram amounts in the urine. Homogentisic acid is oxidized by air on standing and alkalinization to a black pigment. This darkening of urine was easily spotted in former days, when chamber pots were in more frequent use. Today's patients, however, may never recognize the slow darkening of their own urine, as it has a normal, clear appearance when passed. We have come across several patients suspected of alkaptonuria. Obviously, the first and easiest test is to add alkali to the urine and observe eventual darkening. Confirmation of the presence of homogentisic acid can be obtained by GC–MS or, more conveniently, by HPLC with rapid scanning detection, as illustrated in Fig. 5.

Drugs and dietary problems

Laboratories involved in metabolic profiling of body fluids are very aware of the problems encountered with drugs and their metabolites. Much time and unnecessary work have been spent on unknown GC peaks thought to carry diagnostic information, only to discover that the compounds were drug metabolites. The present-day mass spectral library contains a number of drugs and drug metabolites and this, together with years of experience and knowledge of drugs in current use, has minimized the problem. However, occasionally we are still confronted with problems due to drugs, as exemplified below.

During the last 2 years, we have analysed some samples that contained three unusual peaks in large amounts (Fig. 6). The mass spectra of these compounds were very similar, all containing repetitive units of $m/z = 44$. Close examination of the clinical record showed that the patients had received drugs, but a variety of different ones. Further investigations (to be published separately) by high-resolution mass spectrometry and *in vivo* experiments showed that the three large peaks in Fig. 6 were metabolites of glycofurol. This is a new inert, non-toxic solvent for a variety of drugs, including antibiotics, diazepam (valium) and antiepileptics, and is particularly well suited for intravenous administration. The hydroxyl group of glycofurol [$RCH_2(OCH_2CH_2)_nOH$] (R = 2-furyl; $n = 1$–3) is oxidized *in vivo* to give a corresponding set of monocarboxylic acids, and this accounts for the metabolites seen in Fig. 6. Whenever these are present in urine, the inference is that the patient has received some drug intravenously in which glycofurol was used as a solvent.

Another metabolite more and more frequently seen in specimens from patients is saccharin, in our case in the N-methylated form due to diazomethane derivatization. Occasionally, this artificial sweetener may be the dominating urinary metabolite, present in even larger amounts than hippurate (Fig. 7).

CONCLUSION

The past 25 years have demonstrated that chromatographic and mass spectrometric techniques are well suited for the detection of abnormal compounds in blood and urine. These methods are therefore valuable for the diagnosis of a number of

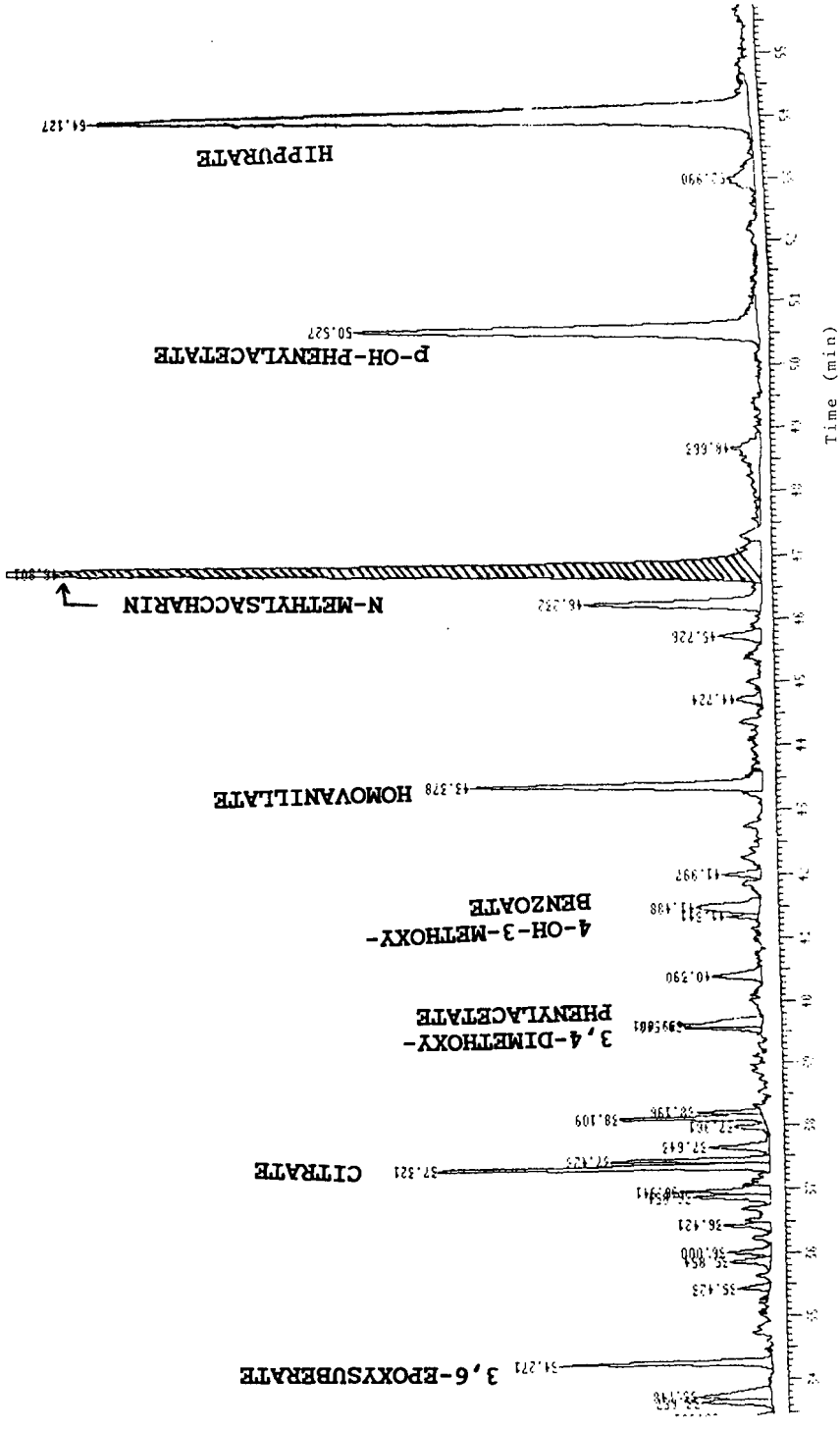

Fig. 7. Organic acid profile (second half) of urine from a person with a large intake of saccharin. Experimental conditions as described in the text and in Fig. 1.

metabolic diseases. A centre for studies of such diseases should, however, also include cell culturing in addition to biochemical techniques, such as enzyme assays, in its analytical repertoire. The question often raised is whether modern DNA technology will make chromatography obsolete for diagnostic purposes. One should realize, however, that DNA technology is as yet suitable for diagnosis only in situations where the family already has had a child with a known disease. The DNA methods are therefore particularly appropriate for prenatal diagnosis. The chromatographic methods, in contrast, can diagnose close to half of all 250–300 metabolic diseases recognized today without a prior knowledge of what to look for. It can therefore be predicted that chromatography will continue to be a most helpful tool for diagnosis and for increasing our knowledge about human metabolic diseases.

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CHROMSYMP. 1542

SEPARATION OF PRECOLUMN *ortho*-PHTHALALDEHYDE-DERIVATIZED AMINO ACIDS BY CAPILLARY ZONE ELECTROPHORESIS WITH NORMAL AND MICELLAR SOLUTIONS IN THE PRESENCE OF ORGANIC MODIFIERS

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SUMMARY

Isoindole derivatives of amino acids, formed through a well-established reaction with *o*-phthalaldehyde, have been effectively resolved by capillary zone electrophoresis and detected fluorimetrically. Relative retention of the formed derivatives is strongly influenced by added detergents and organic modifiers. Analytical reproducibility has also been assessed.

INTRODUCTION

o-Phthalaldehyde (OPA) has been commonly used as a precolumn derivatization agent for the high-performance liquid chromatographic (HPLC) analysis of amino acids[1–5]. In the presence of a reducing agent, such as 2-mercaptoethanol or ethanethiol, OPA reacts specifically with primary amine groups to form highly fluorescent substituted isoindoles. The advantages offered by OPA include its relatively specific and strong fluorescence, rapid reaction with amino acids, and ease of use. However, a major drawback is the instability of OPA-derivatized amino acids, which has reduced its utility in separation methods which require relatively long analysis times. The development of a rapid, efficient, and sensitive separation system is seen as a needed improvement in the area of amino acid analysis.

Capillary zone electrophoresis (CZE) has been developed[6,7] as an effective, high-efficiency separation technique for both small ions[6–11] and macromolecules[12]. To extend the application of CZE to the analysis of neutral species, Terabe *et al.*[13] introduced a variant which is based on the differential distribution of sample species between a mobile phase (electroosmotically pumped) and a micellar phase, formed by adding a surfactant which is retarded due to the electrophoretic effect. This technique has also been termed "micellar electrokinetic capillary chromatography" (MECC) by Burton *et al.*[14] Both CZE and MECC combine the desirable features of high separation efficiency (often on the order of 10^5 theoretical plates) and short analysis times.

In the present study, we explore possibilities for the separation of primary amino acids as their OPA derivatives by CZE, using both a normal solution and a micellar solvent system. Investigations dealing with the stability of the derivatized products,

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reproducibility of the electrophoretic profile, linearity, and the effects of pH and organic modifiers on retention characteristics are described. The application of this method to the separation and analysis of a protein hydrolysate is also demonstrated.

EXPERIMENTAL

Apparatus

A CZE system, similar to that described by Jorgenson and Lukacs[7], was constructed in-house. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A., 50–100 cm × 50 μm I.D., 187 μm O.D.) were suspended between two electrodes, immersed in reservoirs filled with the same buffer solution, all contained within a safety interlock box. The high voltage was provided by a d.c. power supply delivering 0–30 kV (Spellman High-Voltage Electronics, Plainview, NY, U.S.A.). Fluorescence detection was performed with a Model FS950 Fluoromat detector (Kratos, Ramsey, NJ, U.S.A.), with an excitation wavelength of 365 nm and fluorescence emission measured with a 418-nm cut-off filter. An on-column optical cell was made by removing the polyimide coating on a short section of the fused-silica capillary.

Chemicals

All amino acids, proteins, and sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, U.S.A). Ethanethiol and OPA were obtained from Fluka (Ronkonkoma, NY, U.S.A.). Methanol and tetrahydrofuran (THF) were of HPLC grade. Sodium acetate and boric acid were of analytical-reagent grade.

Capillary cleaning and injection procedures

To maintain a high surface charge density on the inner surface of the capillary and create a strong electrokinetic flow, capillary cleaning procedures are necessary[12]. The capillary was initially flushed with distilled water for *ca.* 30 min, followed by 1.0 *M* KOH and 0.1 *M* KOH for 30 min each. The capillary was then rinsed thoroughly with distilled water and equilibrated with the buffer solution to be used for the separation. All cleaning procedures were carried out by using a vacuum pump to draw the liquid through the capillary.

Sample injection was accomplished by hydrodynamic introduction. The high-voltage end of the capillary was dipped into the sample vial, which was raised to a level higher than the other end of the column and maintained for a certain length of time. The end of the capillary was then returned to the buffer reservoir, and high voltage was applied to start the electrophoretic run.

Derivatization and protein hydrolysis procedures

The OPA–ethanethiol derivatizing solution was prepared by dissolving 5 mg of OPA in 0.45 ml of methanol. To this solution, 5 μl of ethanethiol and 50 μl of borate buffer (0.4 *M* boric acid solution adjusted to pH 9.50 with 1.0 *M* KOH) were added and mixed. This solution should be protected from light and freshly prepared prior to each use.

Stock solutions of each individual amino acid were prepared in a phosphate buffer (pH 7.00) at a concentration of 10 mg/ml. A mixture of amino acids was

prepared by mixing equal volumes of the stock solution of each amino acid. A solution of 2-aminoethanol at a concentration of 1 mg/ml was also prepared and included in the mixture as an internal standard. All solutions were kept frozen when not in use.

Protein samples were hydrolyzed by placing 20 μl of protein solution (1.0 mg/ml) into a hydrolysis tube and adding 0.5 ml of constant-boiling HCl. The tube was sealed under vacuum after 30 min and heated at 110°C for 24 h. The solution was lyophilized and the residue was dissolved in 50 μl of distilled water just prior to derivatization.

Derivatization of the amino acids was performed by mixing an aliquot of amino acid standard solution or protein hydrolysate solution (2–5 μl) with an appropriate volume of internal standard solution, 0.4 *M* borate buffer, and 2–5 μl of the OPA–ethanethiol derivatizing solution. The mixture was allowed to stand at room temperature for 1–2 min prior to injection. Approximately 2–20 nl of sample solution were injected for analysis.

RESULTS AND DISCUSSION

OPA, in the presence of either 2-mercaptoethanol or ethanethiol, reacts rapidly with primary amino acids to form highly fluorescent thio-substituted isoindoles[15,16]. In our initial experiments, OPA–2-mercaptoethanol was used to derivatize the amino acids. However, the fluorescence response obtained following separation of the amino acids by CZE was very weak. The instability of fluorescent products in the OPA–2-mercaptoethanol derivatization reaction has been reported[1,15], and it is known that the substituted isoindole decays readily to an ethylene sulfide polymer and 2,3-dihydro-1*H*-isoindol-1-one. It is possible that the heat generated by the applied high voltage during a CZE separation could accelerate the degradation of the OPA–2-mercaptoethanol fluorescent products, leading to the weak signal. In place of 2-mercaptoethanol, we used ethanethiol in the OPA derivatization reactions and found that the stability of the fluorescent product was considerably improved.

Fig. 1 shows an electropherogram of the OPA–ethanethiol derivatives of four amino acids and 2-aminoethanol, used as an internal standard. We initially used a ternary solvent system of aqueous buffer, methanol and THF, similar to that commonly used in reversed-phase HPLC. A mixture of 0.05 *M* sodium acetate, 15% methanol and 1% THF was found to give good resolution of the OPA-derivatized amino acids, seen in Fig 1. The stability of the derivatized components was ascertained by measuring the peak heights from successive electropherograms, obtained over a period of several hours. It was observed that the derivatized products are stable for *ca.* 5 h, exhibiting no significant decrease in the fluorescence response. This stability, coupled with the feature of short analysis times for CZE, makes it possible to put the same sample through several electrophoretic runs.

Reproducibility of the electrophoretic profile of OPA-derivatized amino acids was investigated to assess the value of CZE for quantitative analysis. The precision of the method was determined by injecting the mixture of amino acid standards used in Fig. 1 five times under the same experimental conditions. The ratio of retention time of the amino acids relative to the internal standard was calculated for each amino acid, and peak heights were measured. The calculated values for the mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) are listed in Table I. Analysis of the data indicates that the average R.S.D. for the retention time ratio is less than 2%, while the R.S.D. for the peak-height ratio is 1.3–4.8%.

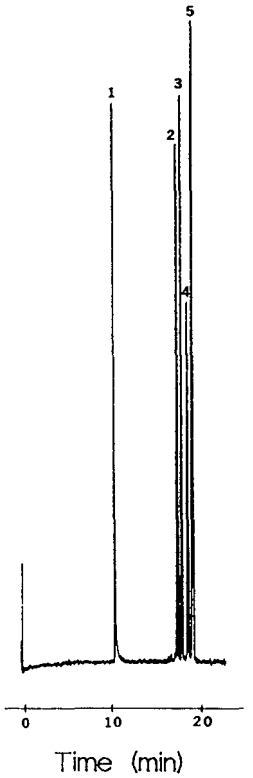

Fig. 1. Electropherogram of OPA–ethanethiol derivatives of four amino acids and an internal standard. Peaks: 1 = 2-aminoethanol (internal standard); 2 = serine; 3 = alanine; 4 = glutamine; 5 = asparagine. Buffer, 0.05 *M* sodium acetate buffer (pH 9.50)–15% methanol–1% THF; capillary, 100 cm × 50 μm I.D. (70 cm to detector); injection time, 2 s; applied voltage, 30 kV.

Presently, two sample introduction techniques are widely used in CZE: the electromigration technique[7] and hydrodynamic suction[10]. The exact amount of sample solution introduced by either technique is not easily determined, but both methods rely primarily on the time of injection. It has been found[17] that the electromigration technique results in some sample discrimination, and the hydro-

TABLE I
REPRODUCIBILITY OF RETENTION TIME AND PEAK HEIGHT

	Peak-height ratio (n=5)			*Retention ratio (n=5)*		
	Mean	*S.D.*	*R.S.D. (%)*	*Mean*	*S.D.*	*R.S.D. (%)*
2-Aminoethanol	1.00	0.00	0.00	1.00	0.00	0.00
Serine	1.14	0.06	4.8	2.03	0.02	1.17
Alanine	1.24	0.05	4.1	2.13	0.03	1.46
Glutamine	0.95	0.02	2.4	2.25	0.04	1.73
Asparagine	1.38	0.02	1.3	2.33	0.04	1.71

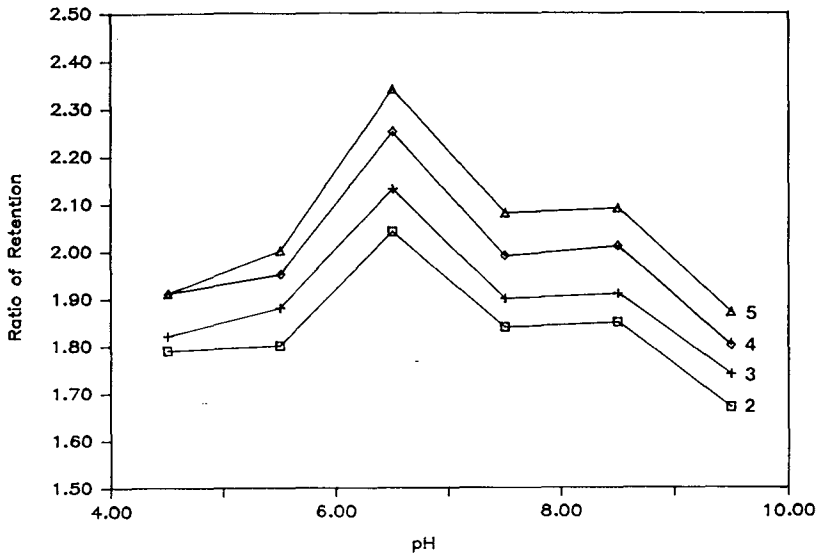

Fig. 2. Dependence of retention ratio on pH. Conditions as for Fig. 1, except for the applied voltage (25 kV). The numbers correspond to the solutes in Fig. 1.

dynamic injection method generally gives more reproducible results[18]. Therefore, the hydrodynamic injection technique was employed throughout this study. The estimated volume of sample solution injected is 2–20 nl, which corresponds to 1.25–12.5 pmol of each amino acid in our study. However, this does not represent the detection limit, which is estimated to be less than 300 fmol of each amino acid. Better reproducibility for both qualitative and quantitative analysis will require more precise control of injection time and further improvement in sample introduction techniques.

An important factor in both CZE and MECC systems is the pH of the buffer solution, since it directly influences the electrophoretic mobility of the solutes and the electroosmotic flow of the buffer solution. The effect of pH on the retention ratio of the OPA derivatives of serine, alanine, glutamine, and asparagine was investigated, as shown in Fig. 2. All four amino acids show the same trends of retention, except at pH 4.50 where both glutamine and asparagine have the same migration time. It is of interest to note that a maximum retention time for these four amino acids was observed at a pH near 7. The migration of charged species in a buffer solution depends simultaneously upon the electrophoretic mobility of the solutes themselves and the electroosmotic flow of the buffer solution. The effect of pH is reflected in a change of the effective charge on the species and the double-layer formed between the capillary wall and buffer solution, which in turn influences the zeta potential and the electroosmotic flow.

The effect of pH on solute migration in a CZE system can also be seen in Fig. 3. These results indicate that pH has very little effect on the electrophoretic mobility of solutes. In contrast, the electroosmotic flow coefficient is more sensitive to pH because of the dependence of double-layer formation on the local electrostatic force. A buffer near pH 7 may result in a decrease of the zeta potential, thereby slowing the

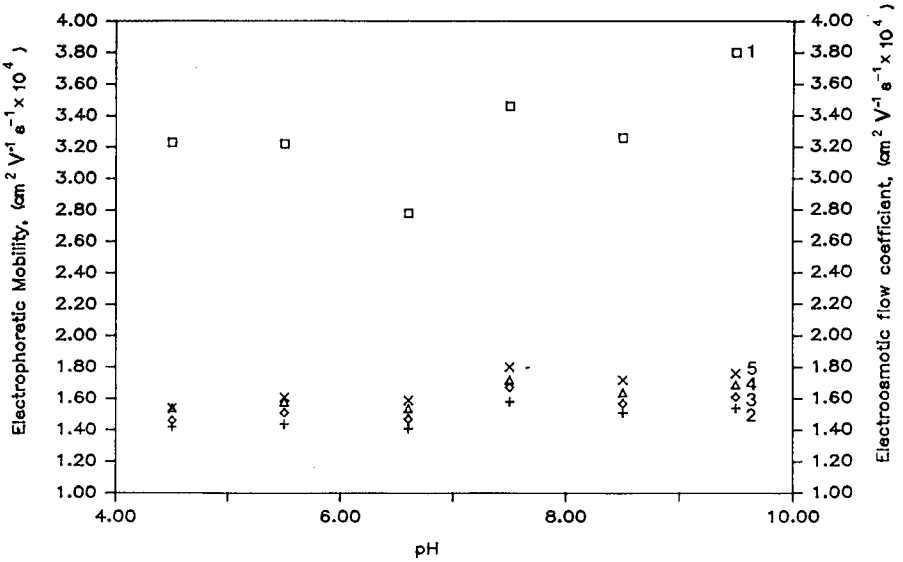

Fig 3. Dependence of electrophoretic mobility and electroosmotic flow coefficient on pH. Conditions and numbers as in Fig. 1. The points for 2-aminoethanol (□) correspond to the electroosmotic flow coefficient (right axis). The points for the remaining components correspond to the electrophoretic mobility (left axis).

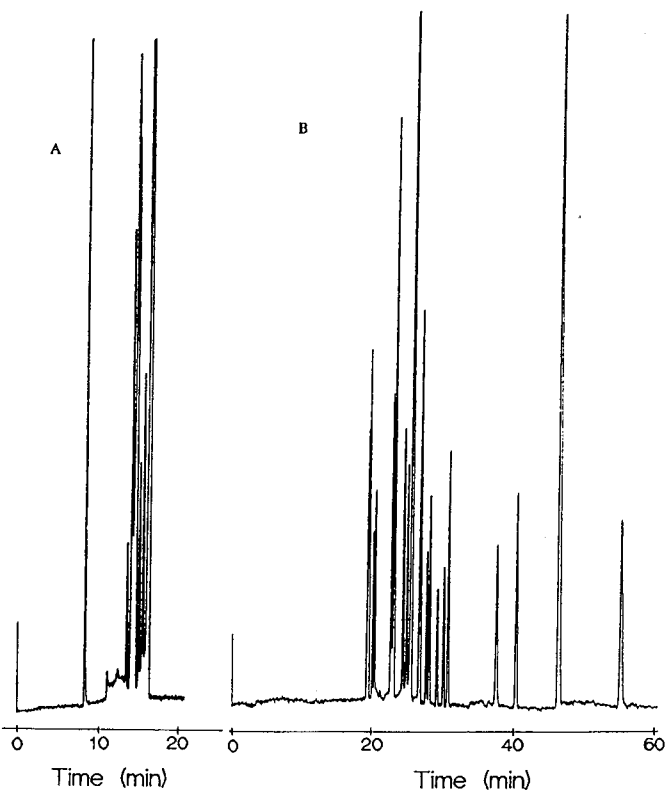

Fig. 4. Electropherogram of a mixture containing eighteen OPA amino acids and an internal standard. Buffer, 0.05 *M* sodium acetate buffer (pH 9.50)–15% methanol–1% THF: (A) without addition of SDS; (B) with addition of SDS (0.05 *M*). Injection time, 15 s. The remaining conditions are as for Fig 1.

electroosmotic flow and the rate of migration of the solutes. A higher pH results in a shorter analysis time, without a significant loss of resolution. A further advantage of using a high pH is the resultant dynamic deactivation of the capillary wall[12], which serves to decrease the adsorption of solutes on the capillary surface. At high pH, the strong electrostatic repulsion between the negatively charged species and the negatively charged capillary walls results in very sharp peaks and high resolution. A pH of 9.50 was found to be optimal for our studies.

Although pH plays an important role in the migration of charged species in CZE, adequate resolution of complex mixtures of OPA-derivatized amino acids is difficult to achieve without further modifications of the buffer system. The addition of SDS, initially utilized for the solubilization and CZE separation of neutral compounds[13], was found to be very effective for improving the resolution of the OPA derivatives. Fig. 4 shows two electropherograms of a mixture containing eighteen OPA-derivatized amino acids, both obtained under the same experimental conditions, except for the addition of SDS. A dramatic improvement in the resolution is noted with the SDS-modified buffer system. The addition of SDS creates a micellar phase within the mobile phase of the buffer. The improved resolution can then be attributed to the effects of distribution of the amino acids between the mobile and micellar phases. In addition, the use of SDS results in an increased dynamic deactivation effect due to the formation of large, negatively charged micelles, which are repelled from the capillary walls. Also, the column capacity is increased with the addition of SDS because of the migration of the micelles in opposition to the direction of the electroosmotic flow. Each of these factors likely plays a role in enhancing the resolving power. It is also evident from Fig. 4 that the internal standard, 2-aminoethanol, which is eluted first

TABLE II
RETENTION PARAMETERS OF OPA DERIVATIVES OF PRIMARY AMINO ACIDS

Peak number	*Amino acid*	*Retention time (min)*	*Retention ratio*
1	Glutamine	16.9	0.425
2	Threonine	17.3	0.435
3	Serine	17.6	0.442
4	Histidine	18.6	0.467
5	Alanine	19.4	0.486
6	Glycine	19.7	0.494
7	Valine	20.4	0.513
8	γ-Aminobutyric acid	20.8	0.522
9	Methionine	21.4	0.533
10	Taurine	21.8	0.548
11	Isoleucine	23.0	0.578
12	Tryptophan	23.9	0.601
13	Leucine	25.2	0.633
14	Lysine	27.6	0.693
15	Glutamic acid	30.2	0.753
16	Arginine	37.4	0.940
17	2-Aminoethanol (internal standard)	39.8	1.00

when SDS is not added to the buffer, migrates more slowly than most of the OPA amino acids when SDS is added. This is due to the fact that OPA 2-aminoethanol is more neutral and hydrophobic, and hence is more easily solubilized by the micellar phase. The slower migration of the micellar phase then results in its longer retention time.

Although the use of a sodium acetate buffer proved satisfactory for the electrophoretic separation of simple mixtures of OPA amino acids, its buffering capacity is limited at the high pH necessary for optimal resolution. For this reason, another quaternary solvent system, consisting of borate buffer, methanol, THF and SDS was employed and found to be very effective in separating all derivatized amino acids. The buffering capacity of the borate buffer is significantly higher than that of the

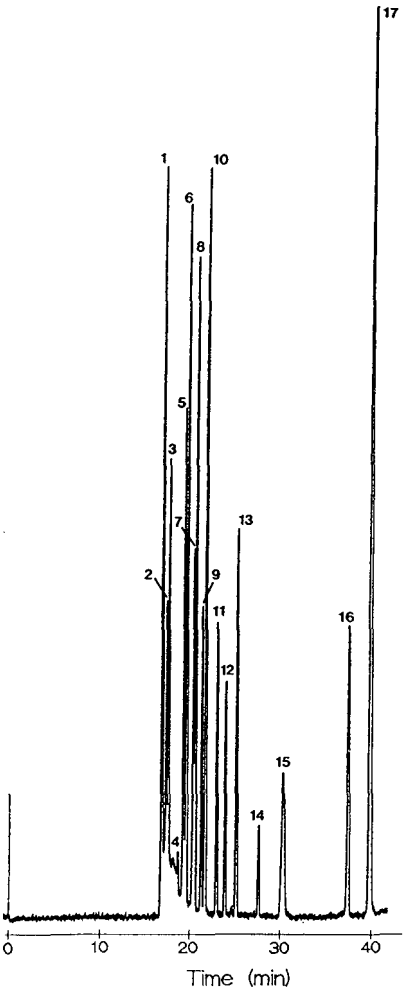

Fig. 5. Electropherogram of seventeen OPA amino acids. Buffer, 0.05 *M* borate buffer (pH 9.50)–15% methanol–2% THF–0.05 *M* SDS; capillary, 86 cm × 50 μm I.D. (53 cm to the detector); injection time, 10 s; applied voltage, 23 kV.

acetate buffer at pH 9.50, resulting in improved resolution. Fig. 5 shows an electropherogram obtained with this buffer system. The peaks in Fig. 5 are identified in Table II, which also lists the retention times and ratios of retention time to the internal standard for the individual components.

Organic modifiers play an important role in CZE separations, particularly for the enhancement of selectivity. Fig. 6 shows the effects of added methanol and THF on the separation behavior of OPA derivatives of glutamine, serine, alanine and glycine in a micellar system. Each of these amino acid derivatives exhibited an increase in

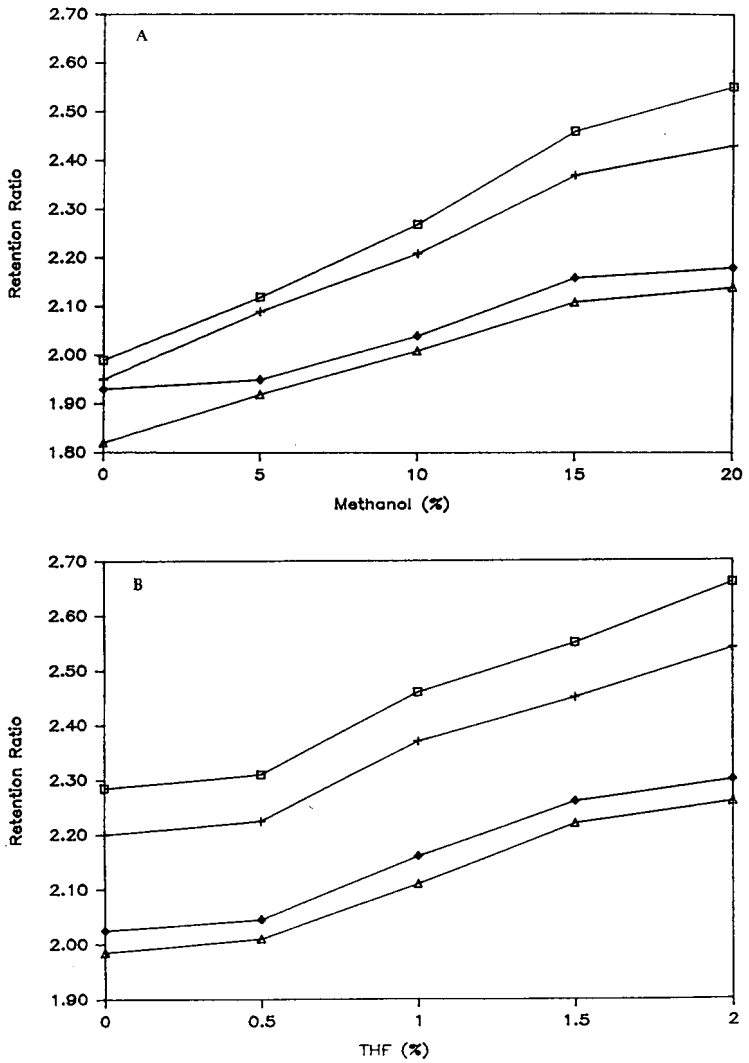

Fig. 6. Effects of organic modifiers on retention: □ = glutamine; + = serine; ◇ = alanine; △ = glycine. (A) Change in methanol concentration (volume percent) in 1% THF; (B) change in THF concentration (volume percent) in 15% methanol. The remaining conditions for both (A) and (B) are as for Fig. 5, except for the applied voltage (20 kV).

retention time as the amount of organic modifier was increased. However, the relative spacing between the components changes with increasing organic modifier, and this improves the selectivity of the separation. This effect is particularly evident in the case of methanol. In our investigations, we found that, when no methanol was added to the buffer system, serine and alanine could not be resolved, while glutamine and glycine were widely spaced. By using a buffer system containing 15% methanol, an evenly spaced baseline separation was obtained for the four components. The addition of organic modifiers was also found to enhance significantly the resolution and selectivity for more complex mixtures. In a mixture of seventeen amino acids, only fourteen peaks were resolved when no methanol was added to the buffer solution. With 5% methanol, sixteen peaks were observed, while with 15% methanol, all seventeen components were resolved (Fig. 5). Fig. 6B shows that the concentration of THF has a much smaller

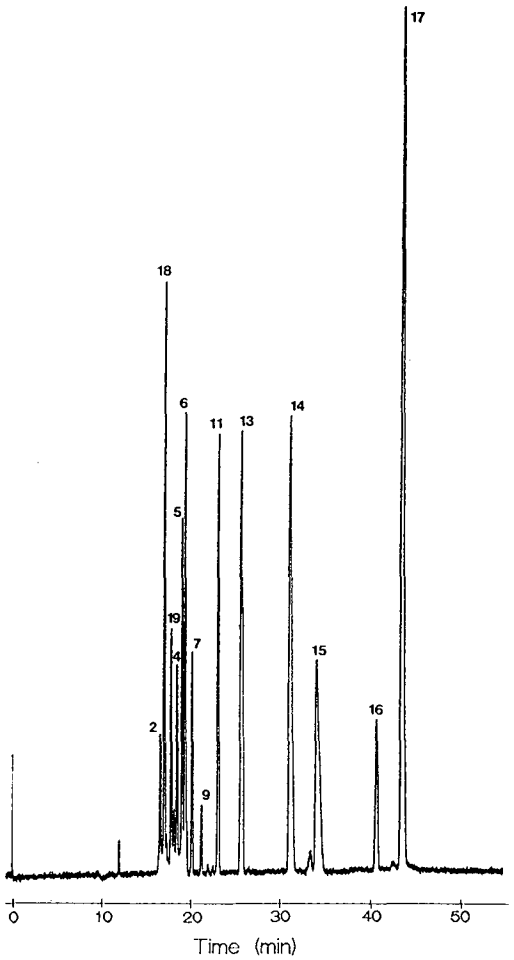

Fig. 7. Electropherogram of OPA amino acids, obtained from the total hydrolysis of cytochrome *c*. Conditions as for Fig. 5, except for the injection time (40 s). The numbers correspond to those in Table II, except that 18 is aspartic acid and 19 is tyrosine.

effect on the relative retention times of the four amino acids. However, it was found that THF is effective in improving the resolution of certain pairs of overlapping amino acids without altering the resolution of the remaining components of the mixture. In our study, an optimum quaternary solution system was found to consist of 0.05 *M* borate buffer, 15% methanol, 2% THF, and 0.05 *M* SDS. The effects of organic modifiers on CZE selectivity can be attributed to their influence on the capillary wall properties, which are related to the zeta potential, enhanced interaction between the solvent and solutes or the micelles and solutes, or a combination thereof.

The application of CZE to the amino acid analysis of a protein hydrolysate is shown in Fig. 7. Cytochrome *c* consists of fourteen different primary amino acids, which are all easily resolved in the quaternary solvent system previously discussed.

CONCLUSION

We have shown that CZE with both normal and micellar solutions and organic modifiers is an efficient and sensitive separation method for OPA-derivatized amino acids. The short analysis times are especially advantageous when dealing with such moderately stable analytes. The high resolution obtainable with CZE indicates a promising application to amino acid analysis in complex biological fluids. Additionally, the selectivity of the separation method can be readily modified with the use of micellar surfactants and organic modifiers. According to a recent report[19], the speed of analysis can be considerably improved by using shorter capillaries, 10 μm in I.D., and thus further improvements are likely in the future.

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SILANEDIOL GROUPS OF THE SILICA GEL NUCLEOSIL: ACTIVE SITES INVOLVED IN THE CHROMATOGRAPHIC BEHAVIOUR OF BASES

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SUMMARY

Fe^{3+} ions in iron(III) chloride-modified silica gels have previously been reported to be bound exclusively to silanediol groups on the surface. A silica gel (Nucleosil) was modified with various amounts of iron(III) and this masking had an influence on the retention of bases. As the iron(III) load increased, the capacity k' factors decreased and the number of plates increased. At a certain iron(III) content saturation occurred and thereafter the k' value remained constant. Only about 40% of the total silanediol groups of Nucleosil influenced retention, which can be explained by the presence of silanediols with different reactivities. Electron paramagnetic resonance measurements of the modified silica gels revealed that Fe^{3+} ions are bound to the silanediol groups as a chelate complex.

INTRODUCTION

The separation of basic compounds by high-performance liquid chromatography (HPLC) on silica gel is often accompanied by long retention times, asymmetric peaks (tailing) and a small number of plates[1,2]. The reason for this abnormal behaviour is not fully understood, but "acidic" silanol groups of the silica gel have been held responsible[1,3]. Addition of cationic modifiers, such as triethylammonium salts, to the mobile phase has been shown to improve the chromatographic behaviour, explained by masking of these active sites[4].

We have doped a silica gel, Nucleosil, with different iron(III) loads and have shown by solid-state nuclear magnetic resonance spectroscopy combined with cross-polarization (CP) and magic angle spinning (MAS) that the Fe^{3+} ions are located exclusively at silanediol groups, because the longitudinal relaxation time, T_1, of the silanediol groups, but not of silanol groups, is greatly reduced, in proportion to the iron content[5]. Moreover, the maximal iron(III) load is equal to the number of silanediol groups, assuming that one iron atom is bound to one silanediol group[5].

In order to investigate whether silanediol groups are the active sites in Nucleosil which influence the retention of bases, silica gels doped with various iron(III) loads were tested by HPLC. We chose a test developed by Daldrup and Kardel[6] for reversed-phase silicas and adapted it to native silica gels under pseudo-reversed-phase conditions.

EXPERIMENTAL

Analysis was performed on a Bruker (Karlsruhe, F.R.G.) LC 31 HPLC system with a Uvikon (Bremen, F.R.G.) 720 LC-VW detector and a Uvikon recorder 21. Electron paramagnetic resonance (EPR) experiments were carried out on a Bruker ESP 300 instrument. Stainless-steel columns (12.5 cm × 4 mm I.D.) were packed by proprietary procedures, using a slurry method.

Packing materials

The silica used was Nucleosil with a particle size of 7 μm and a surface area of 350 $m^2\ g^{-1}$ (Macherey, Nagel & Co., Düren, F.R.G.). Iron(III) chloride hexahydrate (analytical-reagent grade) dissolved in distilled water was used as the dropping solution (pH 3). At pH 3 iron(III) has been found to be completely adsorbed on silica[7]. The iron(III)-modified phases were prepared by shaking 3 g of silica for 1 h in 40 ml of aqueous solutions with different iron(III) concentrations (0.1–0.03 mol/l). The silica was filtered (G4), washed five times with 20 ml of distilled water and dried at 100°C for 12 h before being packed. The iron(III) load was determined at 500 nm photometrically with sulphosalicylic acid according to Marcenko[8].

The mobile phase was acetonitrile–buffer (5 mmol) (5:1, v/v) and the buffer was a stock solution of 6.66 g of potassium dihydrogenphosphate + 4.8 g of 85% phosphoric acid in 1 l aqueous solution, which was diluted 1:10 (pH 3.4). The flow-rate was 1 ml/min and detection was at 220 nm.

Samples

HPLC-grade acetonitrile and other reagents were obtained from Merck (Darmstadt, F.R.G.), except 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) (Aldrich Europe, Beerse, Belgium). The chromatographic sample mixture contained 0.5 $\mu g/\mu l$ of diphenhydramine hydrochloride (DPHA · HCl) and 0.5 $\mu g/\mu l$ of MPPH.

RESULTS

The commercially available Nucleosil contains 0.14 $\mu g/m^2$ of Fe^{3+} as a natural contaminant. Doping of Nucleosil with different iron(III) concentrations resulted in silica gels with iron(III) loads of 1, 5.7, 7.4 and 17.1 $\mu g/m^2$, which were used in the HPLC test. The maximum load of 17.1 $\mu g/m^2$ of Fe^{3+} ions could not be exceeded, even with highly concentrated iron(III) doping solutions.

The sample mixture used contained DPHA · HCl and MPPH. DPHA · HCl is a basic drug that is extremely sensitive to the polar silanol groups[6], whereas the retention of MPPH has been shown to be independent of the polarity of different silica gels[6]. In preliminary experiments, we investigated the influence of the Fe^{3+} modification on the retention of MPPH. The capacity factors (k') were not influenced by the iron(III) load and were therefore used as a reference. The iron(III) load of the Nucleosil has a great influence on the retention time and the number of plates for DPHA · HCl. Increasing the iron(III) concentration results in a decrease in k' values and an increase in the plate number (N) (Table I). These effects are not reversible. Prolonged washing with the eluent for 1 day reduces the iron(III) load by 30% to a stable value, and further washing has no great effect. The k' values increase slightly,

TABLE I

DEPENDENCE OF k' VALUES AND NUMBER OF PLATES, N, OF DPHA · HCl ON THE IRON(III) CONTENT OF IRON(III)-MODIFIED NUCLEOSIL

$[Fe^{3+}]$ ($\mu g/m^2$)	k'	N/m
0.14	1.68	2000
1.00	1.56	3060
5.7	1.22	3650
7.4	1.09	4430
17.1	1.05	7240

but never reach the unmodified level. A plot of ln k' *versus* iron(III) content (Fig. 1) shows saturation kinetics, and this allows the determination of the amount of iron necessary for saturation of the active sites of Nucleosil. Extrapolation of the steepline in Fig. 1 to the abscissa gives a value of 8.85 $\mu g/m^2$ of Fe^{3+} ions, corresponding to 0.159 $\mu mol/m^2$. Even higher iron(III) loads have no greater influence on the retention time. Although Fe^{3+} ions can mask all silanediol groups in Nucleosil[5], only a proportion of the total silanediol groups are actually active sites. Whereas the total silanediol group concentration has been estimated to be 0.37 $\mu mol/m^2$ (ref. 5), the concentration of active sites is 0.159 $\mu mol/m^2$, and therefore only *ca.* 40% of the silandiol groups in Nucleosil influence the retention times of bases, such as those used in the HPLC test.

Native and iron(III)-modified silica gels were examined by EPR in order to determine the configuration of iron(III) bound to the surface. Fig. 2 shows the derivative spectra at 77 K for a native silica gel and an iron(III)-modified phase (5.7 $\mu g/m^2$ of Fe^{3+} ions). The native silica gel shows a weak $g = 4.29$ resonance and a signal at $g = 2.014$, whereas the modified phases show a shoulder resonance at $g = 8.6$,

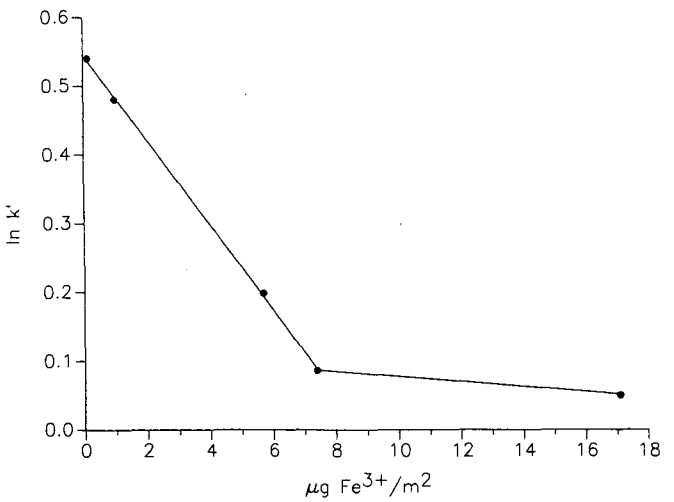

Fig. 1. Plot of the logarithm of k' values of DPHA · HCl *versus* iron(III) content of iron(III) chloride-modified Nucleosil.

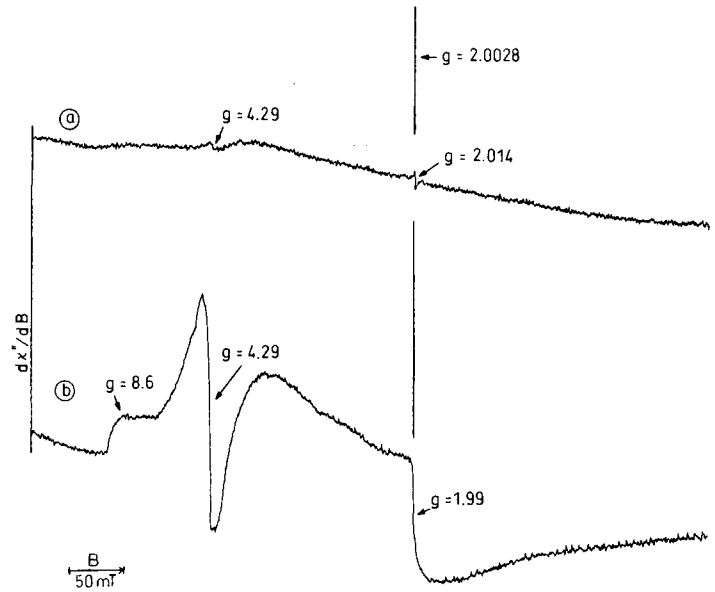

Fig. 2. Derivative of the absorption, $d\chi''/dB$, at 77 K for (a) native Nucleosil and (b) iron(III)-modified Nucleosil (5.7 $\mu g/m^2$ of Fe^{3+} ions).

a signal at $g = 4.296$ and a resonance at $g = 1.99$. Castner *et al.*[9] explained the resonance at $g = 4.29$ and 2.014 occurring in native silica gels as a high spin of the $^6S_{5/2}$ Fe^{3+} ion. These Fe^{3+} ions are isolated and not coupled, occupying sites similar to silicon in the network. After doping, the intensity of the $g = 4.29$ peak increased strongly, and a resonance at $g = 1.99$ appeared. Here, the Fe^{3+} ions are isolated and also not coupled. The resonance at $g = 1.99$ is due to a strongly asymmetric rhombohedrial surrounding for Fe^{3+} ons on the surface[10]. For Fe^{3+} ions occupying silicon sites within the network in a more symmetrical surrounding, the resonance at $g = 1.99$ should be weak[9]. These measurements and the fact that the effects of masking active sites with Fe^{3+} ions are not reversible indicate a strong specific interaction between the Fe^{3+} ions and the silanediol groups. The Fe^{3+} ions could form a chelate complex with the oxygen atoms of silanediols.

DISCUSSION

The retention characteristics of bases in native silica gels under pseudo-reversed-phase condtions are complex functions of the organic solvent and buffer concentrations, and also pH[1]. In the chromatographic test system used, the mobile phase contains a high concentration of organic solvent and the silanophilic interactions are strengthened. DPHA · HCl is protonated in the mobile phase (pH 3.4)[6]. The interaction with active sites can be explained by an ion-exchange mechanism, represented by

$$NR_3 + H^+ \rightleftharpoons NR_3H^+ \qquad (1)$$

$$SiOH \rightleftharpoons SiO^- + H^+ \quad (2)$$

$$SiO^- + NR_3H^+ \rightleftharpoons SiO^-NR_3H^+ \quad (3)$$

The masking of acidic SiO^- groups (eqn. 2) by Fe^{3+} influences the ion-exchange strength, because the number of available SiO^- groups is reduced and the ion-exchange equilibrium constant changes. With this model, the curves in Fig. 1 can be readily explained. In iron(III)-modified silica gels with an iron(III) content below the saturation level, the k' values are influenced by the remaining active silanediol groups. This process can be correlated with the steep branch in Fig. 1. After saturation, all active sites are masked.

Acidic silanediol groups could be among the active sites in both native silica gels and in reversed-phase materials: CP–MAS solid-state nuclear magnetic resonance spectra of chemically modified silica gels revealed that silanediol groups remain present on the surface[12]. Silanol groups could also be active sites contributing to the retention of bases. However, the interaction between active silanol groups and basic compounds cannot be investigated with the test system used, as Fe^{3+} ions bind exclusively to silanediol groups. To determine the contribution of active silanol groups, a reagent specially affecting silanol groups must be found.

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STABILITY CONSTANTS OF HYDRACID–TRIBUTYL PHOSPHATE COMPLEXES IN ALKANE SOLUTION: HYDROCHLORIC ACID AND HYDROGEN CYANIDE

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SUMMARY

The gas chromatographic technique of elution by characteristic point (ECP) has been used to determine the stoichiometric stability constants (K_1) of complexes of both hydrochloric acid and hydrogen cyanide with tributylphosphate (TBP) in hexadecane solution. The study covered the temperature range 25–65°C and the concentration ranges in solution: TBP (0–1.1 *M*) and acids (0–0.3 *M*). Values of K_1 determined ranges between 20 and 120 l mol^{-1} depending on conditions. An extension of gas–liquid chromatographic theory to take into account dilution of solvent and depletion of TBP (due to the large K_1 prevailing) at high acid levels is presented and its validity demonstrated. This will allow correction for purely formal effects to be made in future studies of this type.

The results are shown to be of high accuracy and, hence, both to provide a secure reference base for further studies and to establish ECP as a technique of wide applicability, and relative practical simplicity, for the investigation of systems of large K_1 at finite (practical) concentrations.

Measurements for the acids in hexadecane alone allow estimation of the enthalpy of vaporisation of hydrochloric acid around its critical temperature as 3.8 kcal mol^{-1}, and of monomeric hydrogen cyanide (25–65°C) as 2.4 kcal mol^{-1}. The latter figure implies an enthalpy associated with the hydrogen-bonding polymerisation of liquid hydrogen cyanide of 3.6 kcal mol^{-1}.

INTRODUCTION

The earliest quantitative study of a complexation reaction by a gas chromatographic technique appears to be that of Du Plessis and Spong[1] who determined the stoichiometry of a silver ammine complex via elution of ammonia from a silver nitrate column. Significant advance, however, came with the work of Gil-Av and Herling[2] who derived the equation, for 1:1 complex stoichiometry, linking the infinite dilution partition coefficient (K_R^∞) of a volatile complexer (A) with the stability constant (K_1) and the concentration (c_B°) of complexing agent (B) in the column solvent. Shortly thereafter, Purnell[3], presented a general treatment allowing evaluation of stability

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constants from retention data for complexes of any type and stoichiometry. These equations have formed the basis of the many studies since conducted. Reported work has been entirely confined to the condition of infinite dilution of the volatile ligand and, further, with only a few notable exceptions, such as the work of Schurig *et al.*[4], to weak complexes (K_1 *ca.* 1 l mol^{-1}). There is no reason to be bounded by these constraints as we show later.

The construction of adsorption isotherms from gas–solid chromatographic (GSC) elution data is a process of long standing, having been pioneered by Glueckauf[5], James and Phillips[6] and Gregg and Stock[7] in the 1940s and 1950s. Its more widespread use, however, followed the comprehensive investigations of Cremer[8], Cremer and Huber[9–10] and of Huber and Keulemans[11] who concentrated on the technique later designated "elution by characteristic point" (ECP) by Conder and Purnell[12–15]. The latter developed a general theory of chromatographic retention applicable at all solute concentrations to all forms of chromatography and which took into account all the equilibrium and kinetic processes that contribute to band shape and retention. They characterised a number of variants of technique[15] which could provide partition data and showed how these would be extracted from the raw data. The treatment also indicated the experimental requirements for elimination of some or other of the non-ideal processes that contribute to the elution process.

In the ECP technique, two such processes dominate. First, the compression of carrier gas. In practice, this is minimised, and the associated approximations validated, by use of very small pressure drops (short columns, coarse packing). Secondly, the sorption effect which arises because of changes of localised mobile phase velocity over the solute band and which leads to skewing in the direction of the band front. This process is minimised by working in conditions where the solute partial pressure in the carrier (p) is small (large K_R).

Given these conditions are met, the necessary data for the determination of partition coefficients as a function of sample size (concentration) can be evaluated directly from the chromatograms via the equations:

$$q = n_A S_A / V_L S_t \tag{1}$$

where q (mol. vol.$^{-1}$) is the amount of sample sorbed per unit volume of liquid, and n_A is the number of moles injected. S_A is the area of the chromatographic trace comprising the sum of (i) the rectangle of peak height (h) and length ($t - t_d$), the distance from the dead volume to peak maximum and (ii) the residual area of the trailing side. S_t is the area bounded by the peak itself. For the gas phase, the concentration c is given by

$$c = n_A s\, h / S_t F_c \tag{2}$$

where s is the chart speed and F_c is the fully corrected carrier flow-rate. Obviously consistent units must be used throughout. It is then found that

$$K_R = q/c = c_l/c_g$$

It is an elegant aspect of ECP that, provided n_A is known accurately, detector calibration is unnecessary.

We now turn to an important issue that does not seem to have been addressed previously. In GSC, V_L is replaced in eqn. 1 by adsorbent weight (w_s) which remains constant at all solute concentrations since adsorbent surface area is invariant. In gas–liquid chromatography (GLC), however, the liquid (solvent) volume increases as the amount of volatile sample rises. Thus, K_R will appear to change for reasons that have nothing to do with non-ideality of solution but because of the way in which we define K_R. This must, therefore, be allowed for.

Further, in strongly complexing systems, where solubility is essentially determined by complexation, as the volatile reactant concentration increases at fixed concentration of the complexing solvent, there is progressively less free solvent to dissolve both complex and uncomplexed ligand. We show later how these effects can be allowed for.

Tributyl phosphate [TBP = $(BuO_3)PO$] is remarkable in that it complexes with a huge range of substances, sometimes very weakly but, more often, very strongly. It is also soluble in many organic liquids whilst also dissolving water via formation of a discrete complex or complexes. In consequence, it is very widely used as an extractant in counter-current and related purification processes. Since it does not have a dissociable proton it can act as a Bronsted base and form, as the ligand, very strong complexes with acids. In aqueous solutions, it is thought[16] that hydrogen chloride, for instance, is bound in complexes of the type $[(BuO)_3PO]HCl \cdot (H_2O)_3$ or $[(BuO)_3PO]_2HCl \cdot (H_2O)_6$. In wet organic phases, however, the species present is thought[17] to be the ion pair $[H(H_2O)_4]^+[X(TBP)_x]^-$, X typically being Cl, Br, etc. In anhydrous systems the evidence[16] is clear that the TBP–acid stoichiometry is 1:1 which means that the complex may be represented as $[(BuO)_3PO]HCl$. Despite the widespread use and interest in TBP as an extractant, few measurements of the stability constants of its complexes have been reported. A notable exception[18] is the GLC based study of TBP–alcohol complexes which yielded, effectively at infinite dilution, values for C_1–C_9 alcohols in the range 4.3–0.2 l mol^{-1}. These are, of course, quite small.

The aims of this work are thus threefold.

(1) To illustrate how the GLC technique may be applied to complexing systems of very large K_1.

(2) To validate the use of the technique at finite volatile ligand concentrations.

(3) To generate reliable values of K_1 for TBP–hydracid complexes as a basis for more extensive studies relating to systems of practical interest.

THEORY

Dilution effect on K_R

Let us take the simplest situation, that in which a solute (1) dissolves in solvent (2) with an activity coefficient (γ) that is concentration independent. Then we may write

$$p_1 = p_1^\circ \gamma_1 \frac{n_1}{n_1 + n_2}$$

where p_1 and p_1° represent the partial and saturation vapour pressures and n the number of moles.

Dividing top and bottom by $\Sigma V = V_1 + V_2$ gives, assuming ideal gas behaviour,

$$p_1 = p_1^\circ \gamma_1 c_1^l \frac{\Sigma V}{n_1 + n_2} = c_1^g RT$$

where c represents concentration in the indicated phase. Hence, the partition coefficient, K, is given by

$$K = c_1^l / c_1{}^g = \frac{n_1 + n_2}{\Sigma V} \frac{RT}{\gamma_1 p_1^\circ}$$

At infinite dilution of 1, as is well known,

$$K^\infty = \frac{RT}{\bar{V}_2 \gamma_1 p^\circ}$$

Hence, with γ_1 constant,

$$K = K^\infty \frac{n_1 + n_2}{\Sigma V} \bar{V}_2$$

Substituting throughout, $V/\bar{V} = n$, $V/\Sigma V = \varphi$ and recognising that $\varphi_1 + \varphi_2 = 1$,

$$K = K^\infty \left[1 + \varphi_1 \left(\frac{\bar{V}_2}{\bar{V}_1} - 1 \right) \right] \tag{3}$$

Thus, if $\bar{V}_2 > \bar{V}_1$, as will usually be the case in GLC, as φ_1 increases, K will appear progressively to exceed K^∞. Thus, where γ values are constant, the variation of K will superficially imply concentration dependent non-ideal interaction where none exists. The factor in parentheses should therefore be used to correct K for this effect if the real effects of concentration on γ_1 (and/or γ_2) are to be isolated. Finally, we note that where, as in this work, the liquid complexer is dissolved in an unreactive solvent (diluent), $\bar{V}_2$ is the molar volume of the mixture, which can be directly measured.

Solvent depletion in complexation

Having already dealt with the dilution effect we can treat this problem initially as one in which the solvent system maintains constant volume.

Consider first the simple situation of a system of 1:1 stoichiometry described by

$$\begin{aligned} &\overset{K_R^\circ}{\rightleftharpoons} \\ \mathrm{A}(g) &\rightleftharpoons \mathrm{A}(l) \\ &\overset{K_1}{\rightleftharpoons} \\ \mathrm{A}(l) + \mathrm{B}(l) &\rightleftharpoons \mathrm{AB}(l) \end{aligned}$$

It is well known[2,3] that at infinite dilution, the effective partition coefficient K_R is given by

$$K_R = K_R^\circ [1 + K_1 c_B^\circ] \tag{4}$$

where c_B° is the initial concentration of liquid-phase complexer. But, as the amount of A rises in the system, more and more of B is removed as AB, and since the model we are considering is one in which we vary the amount of A added and maintain c_B° at some fixed value, this must have an effect on K_R since the prevailing concentration, c_B, is not constant. At constant system volume

$$\Sigma V = V_{A(l)} + V_{B(l)} + V_{AB(l)} + V_{dil}.$$

When complexing is strong $V_{A(l)}$ is, of course, very small.
Now,

$$\Sigma n_{A(l)} = n_{A(l)} + n_{AB(l)}$$

whence, dividing through by ΣV,

$$\Sigma c_{A(l)} = c_{A(l)} + c_{AB(l)}$$

But

$$c_{AB(l)} = K_1 c_{A(l)} c_{B(l)},$$

while,

$$c_{B(l)} = c_{B(l)}^\circ - c_{AB(l)}$$

and correspondingly,

$$c_{AB(l)} \gg c_{A(l)}$$

so, with little approximation,

$$\Sigma c_{A(l)} = c_{A(l)} + K_1 c_{A(l)} [c_{B(l)}^\circ - \Sigma c_{A(l)}]$$

Dividing now by $c_{A(g)}$ we have

$$K_R = K_R^\circ [1 + K_1 (c_{B(l)}^\circ - \Sigma c_{A(l)})]$$

When $\Sigma c_{A(l)}$ is small, this reduces to eqn. 4. Otherwise, we have

$$K_R = K_R^\circ [1 + K_1 c_{B(l)}^\circ] - K_R^\circ K_1 \Sigma c_{A(l)} \tag{5}$$

Thus, in the absence of a dilution effect, we would expect a linear plot (of negative slope) of K_R against $\Sigma c_{A(l)}$, the latter is, of course, the quantity directly measured in ECP. We note that in the circumstances prescribed, the intercept is the value of K_R at some value of $c_{B(l)}^\circ$ at infinite dilution of A while the slope is the negative value of that of the corresponding plot also at infinite dilution, *viz.* $K_R^\circ K_1$ (eqn. 4).

The combined effects

We see immediately that, when $\bar{V}_2 > \bar{V}_1$, the dilution effect (eqn. 3) increases K_R whereas the depletion effect (eqn. 5) reduces it. When both effects occur, as must be the case, we would then anticipate that a plot according to eqn. 5 would deviate in an upward direction from its initial linearity, *i.e.* the equation would appear to fail for purely formal reasons. Only if there were dramatic changes in the various activity coefficients would the converse occur.

Within the constraints of the approximations stated earlier, it is evident that we can allow for this by combining eqns. 3 and 5 to give

$$K_R = K'_R [1 + \varphi_1 (\frac{\bar{V}_2}{\bar{V}_1} - 1)] \quad (6)$$

where K'_R is the value of K_R to be anticipated via eqn. 5, that is, when the dilution effect is neglected.

With this treatment we are now in a position to correct our data for the formal effects occurring at finite concentration of solute A in GLC when complexing is strong.

EXPERIMENTAL

GC measurements were made using a purpose-built high precision instrument. The system employed a Porter gas flow controller (Hatfield, PA, U.S.A.) in conjunction with a two-stage pressure regulator using helium as carrier gas. To reduce problems of potential reaction of the solutes within the detector, a dual thermister katharometer, operated at just above the column operating temperature, was employed.

The column of 1 m × 4 mm I.D. stainless steel packed with 40–60 mesh coated particles allowed operation at a pressure drop across the column of no more than 150 Torr while providing an acceptable number of theoretical plates ($N > 500$). Pressure drops were measured with a mercury manometer and flow-rates with a soap-film meter making the appropriate corrections for the saturation vapour pressure of the water. The eluent was scrubbed of the hydracid by employing an alkali trap which was replaced at regular intervals.

The injector and column were immersed in a thermostatic water bath equipped with a Techne Tempette (Cambridge, U.K.) TE-8D thermoregulator which provided a temperature stability of 0 ± 0.01°C and temperatures measurement to within $\pm$ 0.1°C.

To minimise possible interfacial adsorption of the solutes at the solid support, the highly silanized support Chromosorb GAW-DMCS (Jones Chromatography, Llanbradach, U.K.) was used. Column packings containing from 2 to 7% (w/w) of liquid phase (TBP in hexadecane) were prepared by the rotary evaporator technique with dichloromethane as the slurry solvent. The dried, free-flowing powders were then packed into precoiled columns with the aid of aspiration and gentle tapping. The phase loadings were determined both by initial weighing and by replicate thermogravimetric analysis with no discrepancy seen between the two measurements.

The densities of the TBP and hexadecane over a range of temperatures are listed in the literature[19,20]. Those of their mixtures were measured directly and found to be

a simple arithmetic volume average of the values for the individual components, indicating a negligible excess volume of mixing. The density equation used was

$$\rho = (0.9964 - 0.00807t)\varphi_{TBP} + (0.7879 - 0.00688t)\varphi_{C16}$$

where t is in °C and φ is the volume fraction.

Solutes were injected through a PTFE coated silicone septum via a Hamilton 1000 series gas-tight syringe. Samples were injected either as headspace samples from aqueous solutions or as ether solutions, with sample sizes ranging from 1 to 100 μl.

RESULTS

Fig. 1 illustrates typical chromatograms obtained with different sample sizes of hydrogen cyanide eluted from a column of TBP–hexadecane (30:70 v/v) at 25°C. The peaks obtained are ideally suited to ECP analysis since at all levels the diffuse trailing edges of the peaks share a common envelope whilst at all but the lowest levels the leading edges are essentially vertical. This conformity establishes that the correct experimental conditions for ECP are operative. It implies also that liquid and solid surface effects are absent, a fact established by use of columns of different solvent loadings which showed no difference in values of K_R.

Fig. 2 shows a comparable set for hydrochloric acid eluted from TBP–hexadecane at 45°C. Here we see a high degree of conformity down to some lower sample level beyond which retention increases. As was said earlier, in the presence of water, hydrochloric acid may complex in more than one way and the indications in the literature are that water–HCl–TBP complexes are stronger than are those of HCl–TBP. Appropriate experiments established that this was the case, at sufficiently

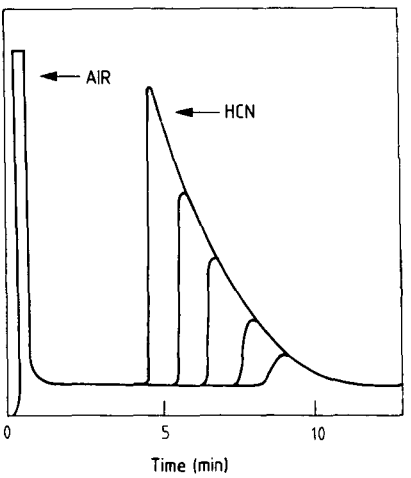

Fig. 1. Elution chromatograms of 0–10 ml samples of headspace gas over aqueous HCN solutions. Column: 1 m × 4 mm I.D. stainless steel packed with 7% (w/w) of 30% (v/v) TBP in hexadecane on 40–60 mesh (ASTM) Chromosorb-G (AW-DMCS). Fully corrected flow-rate of He, 40.4 ml min^{-1}; elution temperature, 25°C.

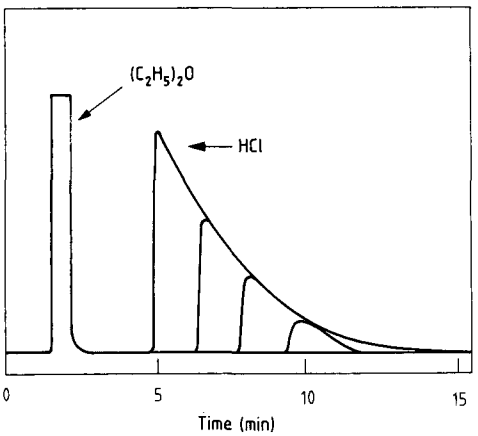

Fig. 2. Elution chromatograms of 0–100 μl of 0.1 *M* solution of HCl in $(C_2H_5)_2O$. Column: 1 m × 4 mm I.D. stainless steel with packing as in Fig. 1. Fully corrected flow-rate of He, 40.2 ml min^{-1}; elution temperature, 35°C.

high water levels the hydrochloric acid peak was followed by a near-plateau extending to long retention times. Intensive drying failed, as is seen, to eliminate the tail completely, although it diminished further with increasing temperature. Where necessary, the extrapolated trailing side of the curve was used to evaluate K_R at low levels of hydrochloric acid and, as seen later, this provided highly compatible data.

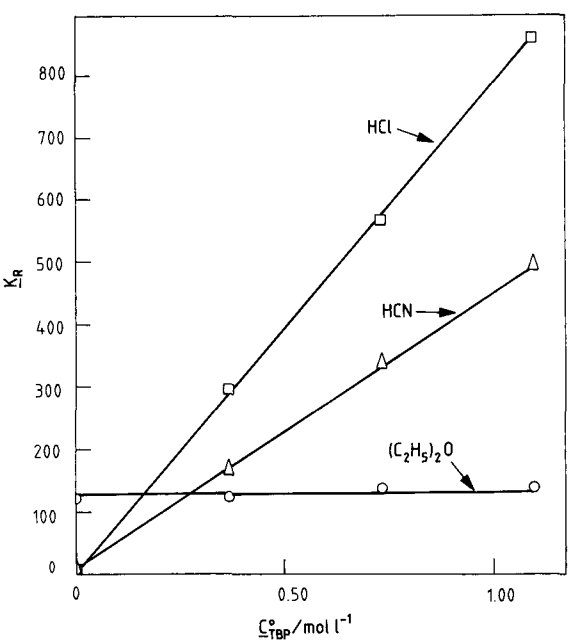

Fig. 3. Plots of infinite dilution partition coefficients between TBP–hexadecane and the gas phase, as a function of TBP concentration, for HCl, HCN and $(C_2H_5)_2O$. Elution temperature, 25°C.

Infinite dilution studies

In situations where small samples provided adequate peak size for direct measurement, K_R could be evaluated via the basic equation, $K_R = V_N/V_l$, where V_N is the fully corrected net retention volume and V_l is the solvent volume. For skewed peaks, an adequate estimate of V_N is derived, following Littlewood[20], from

$$t_R = t_T + t_F - t_{max}$$

where t_T is the time of intersection at the baseline of a tangent to the leading edge, t_F that for the trailing edge and t_{max} is the peak maximum retention time. In many experiments, particularly at higher TBP concentration, retention was so great that peak broadening precluded this approach. Then, the trailing edge envelope for both hydrogen cyanide and hydrochloric acid was extrapolated to the base line.

In Fig. 3 we show the results for 25°C at infinite dilution of hydrochloric acid, hydrogen cyanide and the reference solute $(C_2H_5)_2O$ as the concentration of TBP in hexadecane is varied. The latter was chosen since ethereal solutions of the acids were commonly used in the work. It is important to note that for the hydrochloric acid case large injection volumes were used and extrapolation of the curves excluded the small tail which was seen in the chromatograms. The magnitude of the tail, as already stated, is moisture sensitive but is reduced at high hydrochloric acid concentrations and

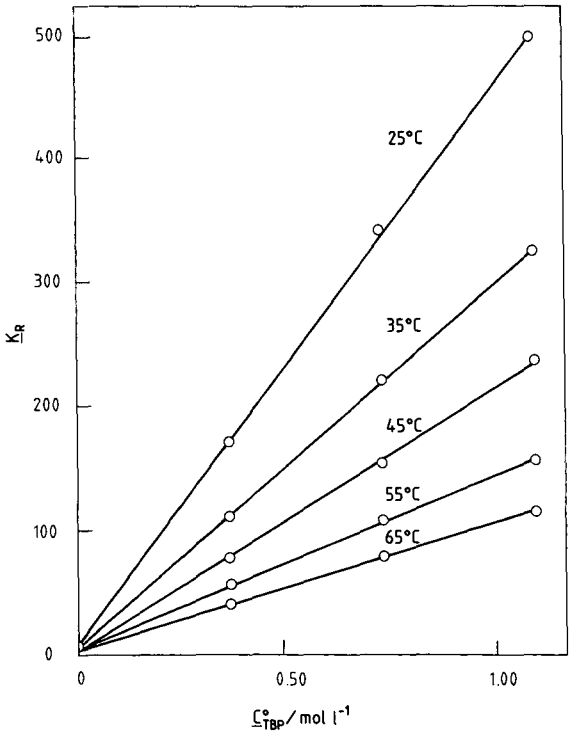

Fig. 4. Plots for HCN as in Fig. 3 for elution at temperatures in the range 25–65°C.

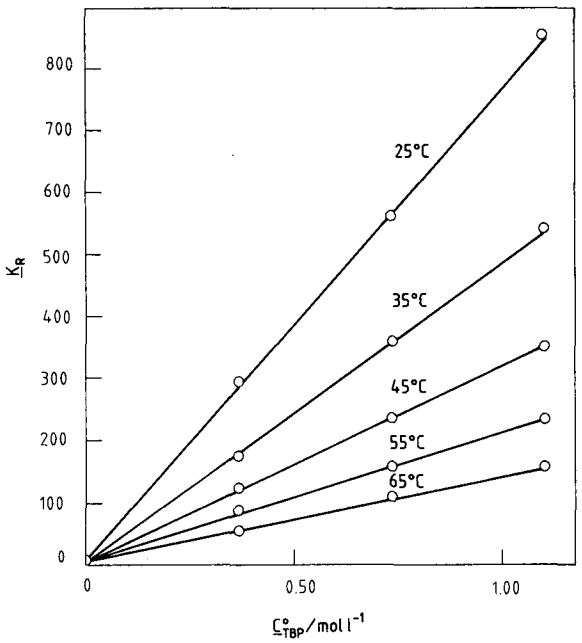

Fig. 5. Plots for HCl as in Fig. 3 for elution at temperatures in the range 25–65°C.

TABLE I

VALUES OF INTERCEPTS (K_R°) AND SLOPES ($K_R^\circ K_1$) FROM PLOTS ACCORDING TO EQN. 4 (FIGS. 4 AND 5) OF DATA FOR ELUTION OF HCl, HCN AND $(C_2H_5)_2O$ FROM TBP–HEXADECANE MIXTURES OF COMPOSITION c°_{TBP} = 0–1.1 mol l^{-1} AND DERIVED VALUES OF STABILITY CONSTANTS (K_1) AND ASSOCIATED THERMOCHEMICAL QUANTITIES, ΔH_1° AND ΔS_1°

Data for K_R° yield enthalpies and entropies of solution of uncomplexed acids: HCl; ΔH° = −8800 kcal mol^{-1}, ΔS = −9.1 cal mol^{-1}K^{-1}: HCN; ΔH° = −2430 kcal mol^{-1}, ΔS° = −3.9 cal mol^{-1}K^{-1}.

Solute	*t(°C)*	K_R°	$K_R^\circ K_1$ *(l mol^{-1})*	K_1 *(l mol^{-1})*	$-\Delta H_1^\circ$ *(kcal mol^{-1})*	$-\Delta S_1^\circ$ *(cal mol^{-1}K^{-1})*
HCl	25.0	6.4	773.8	120.9		
	35.0	5.2	481.4	92.6		
	45.0	4.3	329.9	76.7	4.44	5.4
	55.0	3.6	214.3	61.3		
	65.0	3.0	149.4	48.2		
HCN	25.0	8.3	447.0	53.9		
	35.0	7.3	288.5	39.5		
	45.0	6.4	201.1	31.4	4.87	8.5
	55.0	5.7	141.4	24.8		
	65.0	5.1	103.1	20.2		
$(C_2H_5)_2O$	25.0	117.9				
	35.0	88.5				
	45.0	65.3				
	55.0	52.4				
	65.0	41.5				

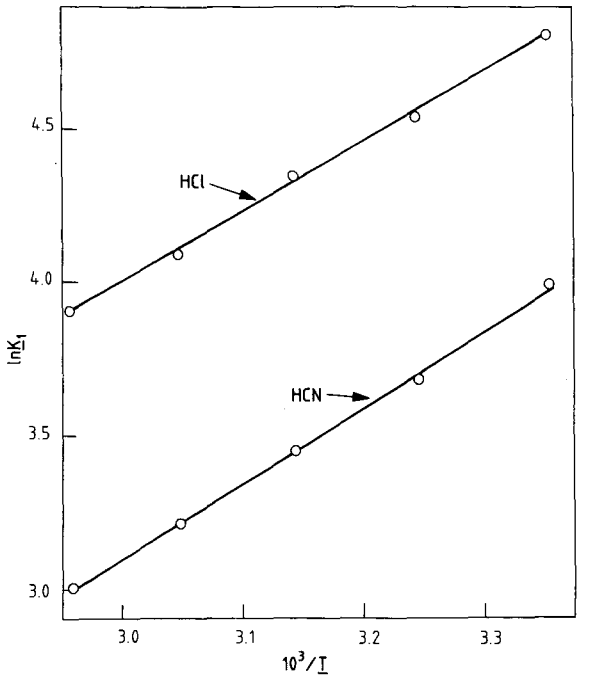

Fig. 6. Van 't Hoff plots of ln K_1 against T^{-1} for HCl and HCN complexes with TBP (1:1) in hexadecane.

elevated temperatures. At very high water concentrations, the tail becomes excessive so that study of hydrochloric acid elution in water saturated systems is precluded by the present method. No such problems were seen with hydrogen cyanide and every effort was made to exclude water from our system in studying hydrochloric acid.

The acid data clearly fall on excellent straight lines in accord with expectation for 1:1 complexes. The data for the ether show little change, perhaps rising a little with increasing c°_{TBP}, and confirm the absence of complexing in this case. Data for both acids over a wide range of temperature and c°_{TBP} are illustrated in Figs. 4 and 5. There is clearly excellent linearity in every instance. From the value of $(K^{\circ}_R K_1)$, having measured K°_R, we evaluate K_1; these data are listed in Table I along with the enthalpies and entropies associated with the acid–TBP–complex equilibrium, the corresponding Van't Hoff plots being shown in Fig. 6.

Eqn. 4 may be rewritten as

$$K_R = K^{\circ}_R + K^{\circ}_R K_1 c^{\circ}_B$$

and since $\Delta G^{\circ} = -RT \ln K$, this may be represented alternatively as

$$K_R = \exp(X) + c^{\circ}_B \exp(Y) \qquad (7)$$

where $(X) = -\Delta G^{\circ}/RT$ for the equilibrium involving solution of uncomplexed acid and (Y) is the sum of this quantity for both processes, solution and complexing. We

TABLE II

COMPARISON OF EXPERIMENTAL AND CALCULATED (EQN. 9) PARTITION COEFFICIENTS (K_R) FOR HCN ELUTED FROM TBP–HEXADECANE MIXTURES OF INDICATED COMPOSITIONS

c_{TBP} (mol l^{-1})	t (°C)	K_R^{exp}	K_R^{calc}	Relative deviation (%)
0.000	25.0	8.6	8.3	+3.5
0.000	35.0	7.1	7.3	−2.8
0.000	45.0	6.4	6.4	0.0
0.000	55.0	5.6	5.7	−1.8
0.000	65.0	5.3	5.1	+3.8
0.367	25.0	170.4	169.3	+0.7
0.363	35.0	111.0	113.9	−2.6
0.360	45.0	77.0	79.0	−2.6
0.357	55.0	56.6	55.9	+1.2
0.354	65.0	40.4	41.1	−1.7
0.733	25.0	341.7	329.8	+3.5
0.727	35.0	220.4	220.9	−0.2
0.720	45.0	154.6	151.7	+1.9
0.714	55.0	107.3	106.9	+0.4
0.707	65.0	79.8	77.0	+3.5
1.100	25.0	497.7	490.4	+1.4
1.090	35.0	320.1	327.5	−2.3
1.080	45.0	222.1	224.3	−1.0
1.071	55.0	157.0	157.6	−0.4
1.061	65.0	113.7	113.1	+0.5

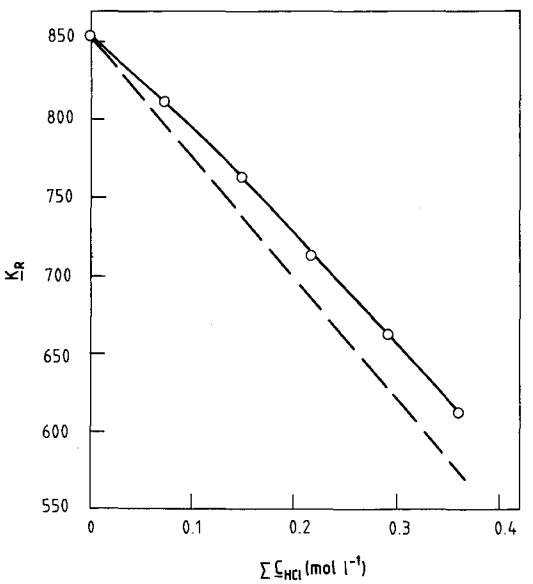

Fig. 7. Typical plot of K_R against total HCl in solution (complexed and uncomplexed) for elution of HCl from column as described in Fig. 1. Elution temperature: 25°C. Broken line corresponds to values of K' (eqn. 5) for these conditions.

TABLE III

COMPARISON OF EXPERIMENTAL AND CALCULATED (EQN. 8) PARTITION COEFFICIENTS (K_R) FOR HCl ELUTED FROM TBP–HEXADECANE MIXTURES OF INDICATED COMPOSITION

c_{TBP} (mol l^{-1})	t (°C)	K_R^{exp}	K_R^{calc}	Relative deviation (%)
0.000	25.0	6.4	6.4	0.0
0.000	35.0	5.3	5.2	+1.2
0.000	45.0	4.2	4.3	−2.4
0.000	55.0	3.4	3.6	−5.9
0.000	65.0	3.1	3.0	+3.2
0.367	25.0	296.2	289.1	+2.4
0.363	35.0	177.4	183.5	−3.4
0.360	45.0	125.4	120.2	+4.1
0.357	55.0	78.3	81.0	−3.4
0.354	65.0	55.0	55.8	−1.5
0.733	25.0	561.9	571.0	−1.6
0.727	35.0	360.1	362.2	−0.6
0.720	45.0	237.3	236.2	+0.5
0.714	55.0	160.4	158.3	+1.3
0.707	65.0	111.0	108.5	+2.3
1.100	25.0	863.4	853.7	+1.1
1.090	35.0	527.5	540.5	−2.5
1.080	45.0	362.8	352.1	+2.9
1.071	55.0	231.0	235.7	−2.0
1.061	65.0	160.5	161.4	−0.6

can, thus, represent both the temperature and c_B° dependence of K_R in a single equation. The terms (X) and (Y) may be calculated either via the estimated values of the enthalpies and entropies of the competing processes or by statistically processing all of the data. Following this latter route we find:

$$\text{HCl}: \quad K_R = c_{TBP}^\circ \exp[(4132/T) - 7.219] + \exp[(1913/T) - 4.565] \tag{8}$$

$$\text{HCN}: \quad K_R = c_{TBP}^\circ \exp[(3679/T) - 6.262] + \exp[(1221/T) - 1.977] \tag{9}$$

In Tables II and III we present the data underlying Figs. 4 and 5, along with the values computed via the above equation. The agreement between the computed and experimental values is clearly excellent.

Studies at finite concentration

Fig. 7 shows a representative plot of the values of K_R measured for elution of hydrochloric acid at 25°C from a column of TBP–hexadecane (30:70 v/v) at acid sample sizes rising high enough to provide an acid concentration in solution of *ca.* 0.4 *M*.

The plot lies above the line for K' and bows upwards exactly as the theory presented earlier demands. In Table III we list the experimental values of K_R for three

temperatures along with those calculated via eqn. 6; the appropriate value of $\bar{V}_1$ for liquid hydrochloric acid being available in the literature[21]. The agreement is, in every case, remarkable in the light of the approximations made. The implications of this finding, that the stoichiometric stability constant is concentration independent, are discussed below.

DISCUSSION

We turn first to the results relating to the infinite dilution work. It is clear from the comparisons illustrated in Tables II and III that there is an excellent internal consistency in the data as expressed by the empirical eqns. 8 and 9. We can, now check the quality of the thermochemical data derived for K_1 since the coefficients within the quantities exp(X) and exp(Y) of eqn. 7 can be calculated from the thermochemical quantities evaluated and cited in Table I. The term exp(X) is given by

$$\exp(X) = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

where the thermochemical quantities relate to the solution of uncomplexed acid, while

$$\exp(Y) = -\frac{\Delta H^\circ + \Delta H_1^\circ}{RT} + \frac{\Delta S^\circ + \Delta S_1^\circ}{R}$$

where now they comprise the sums for both the solution and complexing processes.

Thus, we would predict

$$\text{HCl}: \quad K_R = c_{TBP}^\circ \exp[(4140/T) - 7.250] + \exp[(1910/T) - 4.573] \qquad (8')$$

$$\text{HCN}: \quad K_R = c_{TBP}^\circ \exp[(3668/T) - 6.246] + \exp[(1221/T) - 1.975] \qquad (9')$$

The correspondence between the coefficients of eqns. 8 and 8′ and of eqns. 9 and 9′ is essentially perfect. This finally confirms the quality of both the retention data and the procedures adopted for their extraction from the chromatograms. More importantly, it provides a high degree of confidence in the values of K_1, and of ΔH_1° and ΔS_1°, extracted therefrom.

Having said this it is worthwhile in passing to comment on the values found for the enthalpy of solution of the uncomplexed acids which comprises the sum of the enthalpy of condensation and the excess enthalpy. For hydrochloric acid, the quoted enthalpies of vaporisation are (kcal mol^{-1}) : 4.68(115.3 – 173.3 K); 4.19(122.5 – 309.5 K)[22]; 3.87(189 K = normal boiling point)[23]. It is clear that the value diminishes rapidly with increasing temperature up to the boiling point and may be expected to fall somewhat further as we move towards the critical temperature (324.8 K) which is roughly the centre of our experimental range. Our value of 3.8 kcal mol^{-1} is thus very close to the anticipated value, which implies a very small, possibly zero, excess enthalpy of solution. Our result, therefore, provides a reasonable estimate of the enthalpy of vaporisation of hydrochloric acid around its critical temperature. For hydrogen cyanide however, the situation is quite different. The quoted value[24] for

TABLE IV

COMPARISON OF CALCULATED (VIA K' AND EQN. 6) AND EXPERIMENTAL PARTITION COEFFICIENTS (K_R) FOR HCl ELUTED AT FINITE CONCENTRATIONS IN THE LIQUID, FROM TBP–HEXADECANE (30:70)

t (°C)	c_{HCl} (mol l^{-1})	K_R^{calc}	K_R^{exp}	Relative deviation (%)
25.0	0.073	809.4	810.7	−0.2
	0.149	761.5	762.7	−0.2
	0.218	716.5	714.3	+0.3
	0.292	666.5	662.0	+0.7
	0.361	618.2	613.1	+0.8
35.0	0.062	516.4	517.0	−0.1
	0.129	489.4	488.2	+0.2
	0.196	461.5	454.9	+1.4
	0.273	428.4	417.0	+2.7
	0.334	401.4	382.4	+4.7
45.0	0.071	333.7	336.2	−0.7
	0.147	313.4	317.9	−1.4
	0.219	293.4	296.8	−1.2
	0.306	268.4	275.0	−2.5
	0.375	247.9	258.1	−4.1

293.3 K is 6.0 kcal mol^{-1} compared with our value for the enthalpy of solution of −2.43. This would imply an excess enthalpy of about −3.6 kcal mol^{-1}. In the liquid, hydrogen cyanide is, in fact, polymerised via hydrogen-bonding and so the enthalpy of vaporisation must include the enthalpic contribution arising from the conversion to monomer in the gas phase. In our system hydrogen cyanide, in the uncomplexed form, exists only at very high dilution due to the large K_1 (see below) and so the equilibria involved in the polymerisation process must favour the monomer. We therefore may suggest that the value 2.4 kcal mol^{-1} represents the enthalpy of vaporisation of the liquid monomer. If this is true, the hypothetical boiling point of monomeric liquid hydrogen cyanide is close to 120 K. Correspondingly, the enthalpy associated with hydrogen-bonding is −3.6 kcal mol^{-1}, a very reasonable figure.

Turning to the results at finite concentration of hydrochloric acid we note the remarkable applicability (Table IV) of the dilution–depletion theory advanced earlier. This is an important finding in that it allows a corrective basis for studies at high concentration and K_1 into the future. There are, however, implications of our findings with respect to activities in the system since the theory as presented assumed, for simplicity, no changes in activity coefficients as concentrations varied. Even at the highest concentrations of total acid (Σc_A) in solution there is little uncomplexed acid present on account of the very high values of K_1. For example, when $K_1 = 50\ l\ mol^{-1}$ and $c_B^\circ = 1\ M$, the ratio c_A/c_{AB} is only 0.02. Hence, we might well expect the activity coefficient of hydrochloric acid to remain constant in all the conditions employed here. The concentration of complex, however, rises from zero to as high as 0.3 M or so over the experimental range while that of TBP is correspondingly reduced. It thus appears that neither component exhibits a concentration dependence of the activity coefficient.

There is other evidence on this score too. The infinite dilution data plotted in Figs. 4 and 5 (eqn. 4) are certainly linear and while the acid and complex are present in only trace amounts in these experiments, the concentration of TBP ranges from zero to more than 1 *M*. Linearity of the plots would not be observed if the activity coefficient of TBP were more than trivially concentration dependent. It may be pointed out that, among the many examples of plots according to eqn. 4 in the literature, for a variety of complex types, there are few cases where curvature of such plots is seen. So too is the case in the spectroscopic (UV–VIS) and NMR approaches to K_1 evaluation wherein the data are processed via the Benesi–Hildebrand equation which is closely analogous to eqn. 4. The situation may thus be considered very common, if not general.

Finally, we turn to the results for K_1. The values derived are of the anticipated magnitude and are associated with thermochemical quantities typical of complexation reactions, particularly of the acid–base type involved in formation of the 1:1 complexes which presumably arise via –H——O–P– interaction. Given such a high quality data base it becomes possible either to calculate liquid–liquid extraction coefficients for these acids by combination with similar data for other solvents, or to calculate such data by use of extraction coefficients where these are known. Further, it should also be feasible to determine K_1 values for non-volatile acids such as nitric acid via competitive experiments. More generally, the demonstrated success of both the technique and the theoretical extension offered means that a wide variety of strongly complexing reactions can be studied with some confidence of success. In summary, the opportunity is available to place a large number of extraction processes of importance on a more quantitative and intelligible basis.

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EFFECTIVE SEPARATION OF STEROL C-24 EPIMERS

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SUMMARY

Separation of C-24 epimeric pairs of 24-alkyl sterols has been achieved by reversed-phase high-performance liquid chromatography. Effects of the solvent system and temperature on the separation of these epimers are emphasized. This separation technique was effectively used for the profile analysis of sterol components from *Nervilia purpurea* and *N. aragoana*, demonstrating the characterization and stereochemical determination of the C-24 position of some new sterols. The application of gas–liquid and high-performance liquid chromatography to the separation of C-24 epimers of 23-substituted 24-alkyl sterols, including demethylgorgosterol, gorgosterol and brassinosteroid isomers, is briefly described.

INTRODUCTION

Most terrestrial and marine sterols have an alkyl group at the C-24 position, and in many cases they occur as C-24 epimeric mixtures. The separation and stereochemical assignment of these epimers have important biosynthetic, taxonomic and geochemical implications. Profile analysis of natural sterols is also important in the screening of natural resources as a starting material for the synthesis of biologically active steroids.

Since gas chromatography (GC) was developed as an essential method of steroid analysis[1], the separation of the epimers of C-24 alkyl sterols has always been a challenging aspect. NMR spectroscopy can be used for the assignment of the C-24 stereochemistry[2,3], but it cannot be applied to the analysis of natural sterol mixtures.

Attempts to separate C-24 epimers of steranes[4] and sterols[5] by GC on glass capillary columns have been reported, but the tremendous length of column required and the reproduction of columns that efficiently separated C-24 epimers have not been satisfactory. More recently, high-performance liquid chromatography (HPLC) has been applied to the separation of a number of naturally occurring steroids, and

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many examples of successful applications of HPLC to the separation of natural sterols have been published[6–9].

In 1981, Djerassi and co-workers[10] reported an effective separation of a couple of epimeric pairs of C-24 alkyl sterols with a 22(23) double bond by reversed-phase HPLC on a Whatman Partisil M9 10/50 ODS-2 column. However, under these conditions the corresponding pairs having a saturated side chain could not be separated.

Facile synthetic methods for C-24 stereoisomers of 24-methyl and ethyl sterols were developed in our laboratory[11,12]. This allowed us to attempt the separation of the epimers by chromatographic methods. While our initial attempts by glass capillary GLC were unsuccessful, we have recently found satisfactory HPLC conditions for the separation of the epimeric sterol benzoates on a reversed-phase column (TSK-Gel ODS-120A) (Toyosoda, Tokyo, Japan).

EXPERIMENTAL

Samples

All standard samples were prepared in our laboratory[11,12].

The benzoates were prepared by adding 4 μl benzoyl chloride to a solution of 0.5 mg sterol mixture in 40 μl pyridine. The mixture was stirred overnight at room temperature. After addition of water, the benzoate was extracted with dichloromethane. The benzoate fraction was purified by preparative thin-layer chromatography (TLC)[13].

For the preparation of the trimethylsilyl ethers, 100 μg of a sterol were dissolved in 30 μl trimethylsilylimidazole and the mixture was allowed to stand at room temperature for 30 min. A few μl of this sample were injected into the gas chromatograph.

For the bis-methaneboronate derivatization, a solution of 100 μl methaneboronic acid in dry 50 μl pyridine was added to 100 μg brassinosteroid. The mixture was heated at 60°C for 30 min. Several μl of this solution were injected into the gas chromatograph.

Instruments

A GC-7APrF chromatograph (Shimadzu, Kyoto, Japan) with a solventless inlet system (moving-needle type) and flame ionization detector was generally used. For Fig. 8, a VGA gas chromatograph–mass spectrometer (VG Analytical, Jasco International, Tokyo, Japan) with a split/splitless injector was used.

An ALC/GPC 201D compact-type high-performance liquid chromatograph (Waters, Division of Millipore, Tokyo, Japan) with an UV detector and a Toyosoda column (TSK-Gel ODS-120A) was used.

RESULTS AND DISCUSSION

The reversed-phase HPLC separation of four epimeric pairs of standard 24-alkyl sterols (Table I, **1–8**) was investigated with various solvent systems. Chloroform–acetonitrile, hexane–2-propanol–acetonitrile and dichloromethane–acetonitrile systems were found to be satisfactory solvent combinations (Table I). The most satisfactory separation of the C-24 epimers was achieved with chloroform–acetonitrile

TABLE I

RELATIVE RETENTION TIMES OF BENZOATES OF 24-EPIMERIC STEROLS IN REVERSED-PHASE HPLC

The values are expressed relative to ergosterol benzoate (1.00). Conditions: column, TSK-Gel ODS-120A (250 mm × 4.6 mm I.D.); flow-rate, 0.6 ml/min; temperature, 20°C; detection, 240 nm.

24-Alkyl sterol 3-benzoates	*Relative retention time*		
	Chloroform–acetonitrile (1:4)	*Hexane–isopropanol–acetonitrile (1:3:16)*	*Dichloromethane–acetonitrile (1:4)*
Campesterol (**1**) (24*R*/α)	1.05	1.14	1.06
Dihydrobrassicasterol (**2**) (24*S*/β)	1.13	1.21	1.14
Crinosterol (**3**) (24*S*/α)	0.85	0.90	0.84
Brassicasterol (**4**) (24*R*/β)	1.01	1.04	1.02
Sitosterol (**5**) (24*R*/α)	1.05	1.22	1.08
Clionasterol (**6**) (24*S*/β)	1.08	1.24	1.11
Stigmasterol (**7**) (24*S*/α)	1.04	1.15	1.05
Poriferasterol (**8**) (24*R*/β)	1.08	1.18	1.08
Averaged retention time of ergosterol benzoate (min)	82.0	85.2	111.8

(1:4) at 20°C. The benzoates of the epimeric pairs of 24-methyl sterols, campesteorl (**1**)/dihydrobrassicasterol (**2**) and crinosterol (**3**)/brassicasterol (**4**), were completely separated, as shown in Fig. 1[14]. The benzoates of the epimeric pairs of 24-ethyl sterols, sitosterol (**5**)/clionasterol (**6**) and stigmasterol (**7**)/poriferasterol (**8**), were only partially separated. Separation of these epimers was less effective at an higher temperature. For instance, at 26°C the pairs of 24-ethyl sterols were inseparable, although the 24-methyl sterols were still separated at this temperature.

For the separation of C-24 alkyl homologues, the hexane–2-propanol–acetonitrile (1:3:16) system gave better results than the chloroform–acetonitrile system. For example, a mixture of four 24β-alkyl sterol benzoates (**2, 4, 6** and **8**) gave four peaks with hexane–2-propanol–acetonitrile, whereas only three peaks were observed with chloroform–acetonitrile, as shown in Fig. 2.

Interestingly, the 24α-epimer was eluted before the corresponding 24β-epimer in all the solvent systems examined. Thus, a comparison of HPLC retention times may be helpful in the determination of the C-24 stereochemistry of 24-alkyl sterols having other types of steroid nuclei. The effect of temperature in HPLC must be taken into account in order to achieve the best separation.

The usefulness of this separation technique is illustrated in the analyses of the sterol componetns of *Nervilia purpurea* Schlechter and *N. aragoane* Gaud, which are used as a folk medicine "I-tiam-hong" in Taiwan, shown in Figs. 3 and 4[14]. The excellent separation of C-24 alkyl sterols as well as their C-24 epimers, seen in these figures, enabled us to identify these sterols and their C-24 stereochemistry. ^{1}H NMR spectroscopy and mass spectral (MS) studies of each sterol component isolated in this way gave useful information about their structures. Needless to say, a preliminary separation of a complex sterol mixture into various fractions by argentation chroma-

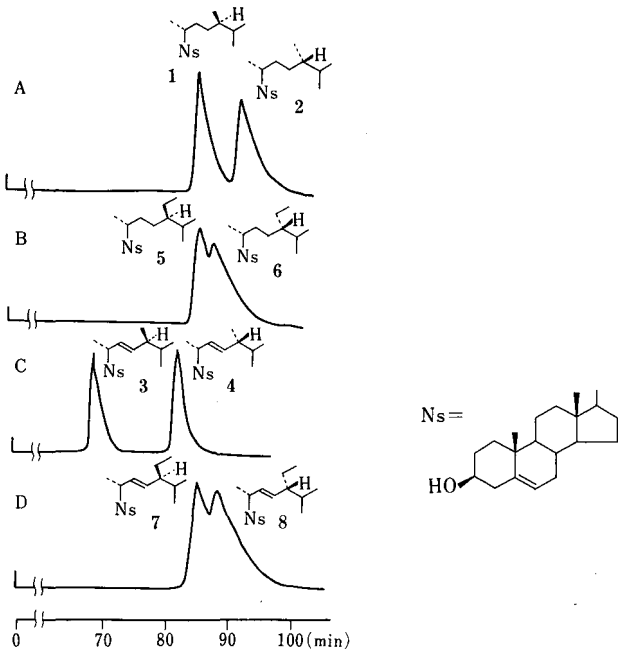

Fig. 1. HPLC separation of the benzoates of C-24 epimeric sterols. (A) Campesterol (**1**)/dihydrobrassicasterol (**2**); (B) sitosterol (**5**)/clionasterol (**6**); (C) crinosterol (**3**)/brassicasterol (**4**); (D) stigmasterol (**7**)/poriferasterol (**8**). Conditions: see Table I; eluent, chloroform–acetonitrile (1:4).

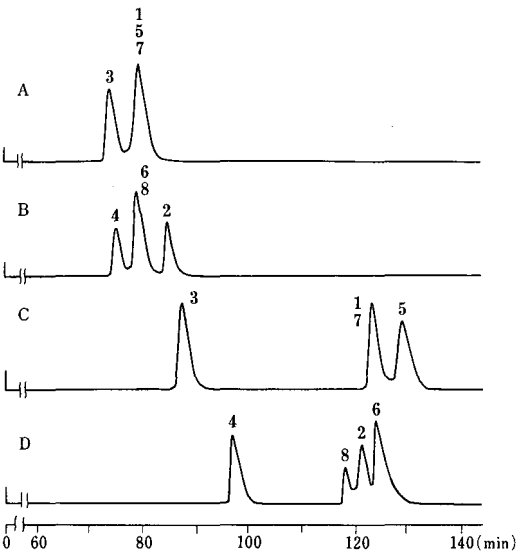

Fig. 2. HPLC separation of the benzoates of 24α- or 24β-24-alkyl sterols. (A) and (C) campesterol (**1**), crinosterol (**3**), sitosterol (**5**) and stigmasterol (**7**); (B) and (D) dihydrobrassicasterol (**2**), brassicasterol (**4**), clionasterol (**6**) and poriferasterol (**8**). Conditions: see Table I; (A) and (B), chloroform–acetonitrile (1:4); (C) and (D), hexane—2-propanol–acetonitrile (1:3:16).

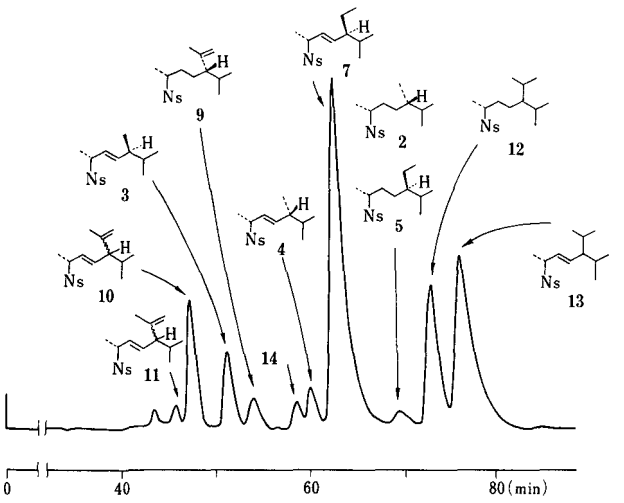

Fig. 3. HPLC separation of the benzoates of the sterol fraction of *Nervilia purpurea*. **9** = (24*S*)-24-isopropenylcholesterol; **10** = nervisterol; **11** = nervisterol isomer; **12** = 24-isopropylcholesterol; **13** = Δ^{22}-24-isopropylcholesterol; **14** = ergosterol. Conditions: see Table I; eluent, chloroform–acetonitrile (1:4).

tography and conventional capillary GLC (or GC–MS) analysis of the fractions may be recommended in certain cases, prior to the present HPLC separation.

A new sterol, 24-isopropenylcholesterol (**9**), was found in *N. purpurea*. For the determination of the C-24 configuration, both epimers were synthesized from fucosterol and separated by HPLC, using chloroform–acetonitrile (1:4) as the eluent. From a chromatographic comparison we concluded that the natural sterol is the 24α-epimer[15]. As expected, the 24α-epimer has a shorter retention time than that of the 24β-epimer. The C-24 configurations of the Δ^{22}-24-isopropenylcholesterols (**10** and **11**) are still not determined because of lack of standard samples.

A group of new 5,10-cyclopropyl-14-methylsterols (**15–20**) was also found in *N. purpurea*. In this group, epimeric pairs of 24-alkyl sterols, *e.g.*, **15/16**, occur in the

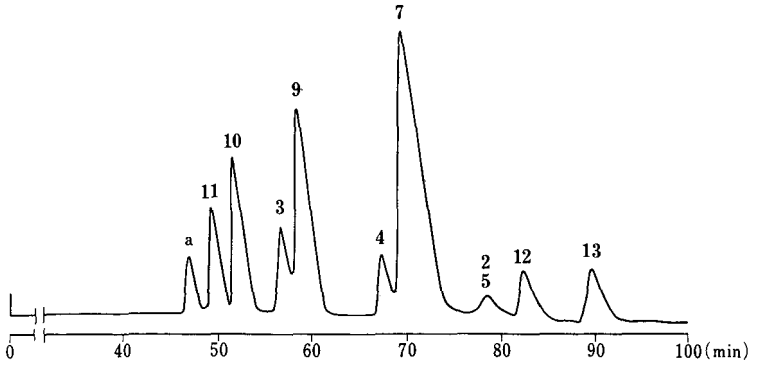

Fig. 4. HPLC separation of the benzoates of the sterol fraction of *Nervilia aragoana*. The sterol structures are shown in Fig. 3. Peak a was not identified. Conditions as in Fig. 3.

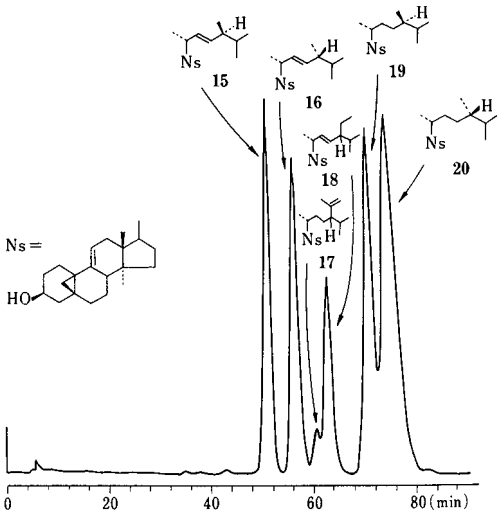

Fig. 5. HPLC separation of the 5,10-cyclopropyl-14-methylsterols from *Nervilia purpurea*. **15** = Cyclonervilasterol; **16** = 24-epicyclonervilasterol; **17** = neocyclonervilasterol; **18** = cyclohomonervilasterol; **19** = dihydrocyclonervilasterol; **20** = 24-epidihydrocyclonervilasterol. Conditions: column, Toyosoda TSK-Gel LS-410A ODS (300 mm × 7.5 mm I.D.); flow-rate, 2.0 ml/min; eluent, hexane–2–propanol–acetonitrile (1:3:16); detection, 225 nm; temperature, 20°C.

same plant. These epimers were also separated by HPLC, as shown in Fig. 5[16–18].

A series of new triterpenes, such as dihydrocycloeucalenol and dihydrocyclonervilol, which have a cyclopropane ring at the C-9/C-10 position and a 4α, 14α-

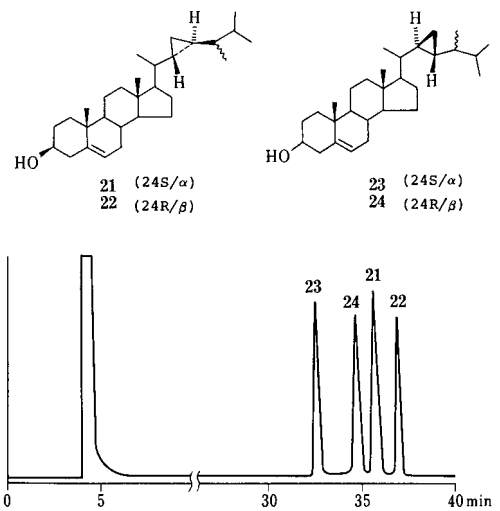

Fig. 6. GLC separation of the trimethylsilyl ethers of desmethylgorgosterol and its stereoisomers. **21** = (22*S*, 23*S*, 24*S*); **22** = (22*S*, 23*S*, 24*R*); **23** = (22*R*, 23*R*, 24*S*); **24** = (22$R, 23*R*, 24*R*) isomer. Conditions: glass capillary column, coated with OV-17 (45 m × 0.25 mm I.D.); oven temperature, 270°C

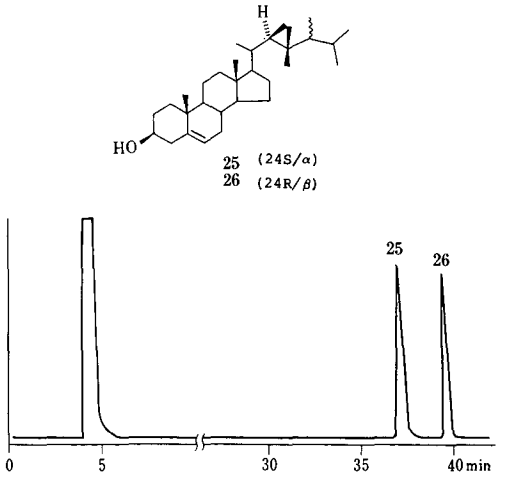

Fig. 7. GLC separation of the trimethylsilyl ethers of gorgosterol (**26**) and 24-epigorgosterol (**25**). Conditions: glass capillary column, coated with OV-101 (25 m × 0.25 mm I.D.); oven temperature, 170°C.

dimethyl group, were isolated from *N. purpurea*. These 24(*R*/α)- and 24(*S*/β)-epimers were also separated by HPLC[19].

In contrast with the aforementioned simple 24-alkyl sterols, C-24 epimers of 24-alkylsteroids which have a functional group at the C-23 position can be separated by GC. Four stereoisomers, (22*S*, 23*S*, 24*S*) (**21**), (22*S*, 23*S*, 24*R*) (**22**), (22*R*, 23*R*, 24*S*) (**23**) and (22*R*, 23*R*, 24*R*) (**24**, natural type) of 24-desmethylgorgosterol, which is a typical marine sterol, having a cyclopropane ring at the C-22/C-23 position, were synthesized in our laboratory[20,21]. These isomers were separated as their trimethylsilyl ethers by GC, using a glass capillary column, coated with OV-17, as shown in

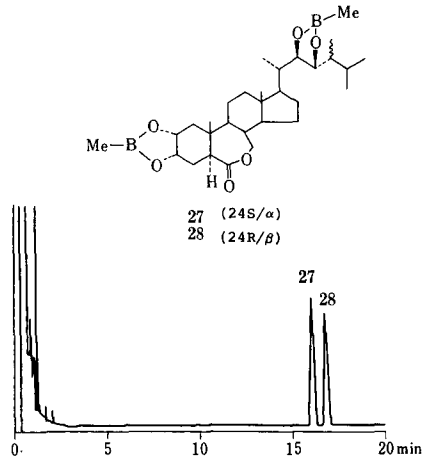

Fig. 8. HLC separation of bis-methaneboronate esters of brassinolide (**27**) and 24-epibrassinolide (**28**). Conditions: fused-silica capillary column, coated with OV-1 (12.5 m × 0.2 mm I.D.); programmed oven temperature 110–320°C (1-min hold at 110°C, then increased at 25°C/min). Me = methyl.

Fig. 6[20]. In this case, the 24(R/α)-isomer had a longer retention time than that of the 24(S/β) isomer. Gorgosterol (**26**) and its 24-epimer (**25**) were separated by the same method, the 24(S/β) epimer being eluted more rapidly than the 24(R/α)-epimer (Fig. 7) [22].

We have recently developed a new micro method for the analysis of brassinosteroids, a new series of steroidal plant growth hormones. In this case, a bis-methaneboronate derivative was used for GLC analysis[23]. Brassinolide (**27**) and 24-epibrassinolide (**28**) were separated as this derivative by a fused-silica capillary column, coated with OV-1 (Fig. 8). This method was applied to the identification of 24-epibrassinolide in the bee pollen of the broad bean, *Vicia faba* L[24].

HPLC with UV detection can be applied to the microanalysis of brassinosteroids. In this case, bis-naphthaleneboronate derivatives of brassinosteroids were employed in chromatography on a Shimadzu Shim-pack CLC-ODS (150 mm × 6.0 mm I.D.) column and 76% aqueous acetonitrile was the eluent. Unfortunately, under these conditions the derivatives of brassinolide and its 24-epimer were not separated[25].

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CONTEMPORARY LIQUID CHROMATOGRAPHY COLUMN DESIGN

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SUMMARY

The development of chromatographic column theory over the last thirty years is described and the impact of the plate theory and rate theory on column design discussed. The concept of the reduced chromatogram is employed to develop equations for optimum column length, optimum column diameter, optimum particle size and minimum analysis time. The equations are used to identify the practical limits of column design and column performance, and the design of a family of columns for general liquid chromatography analysis is recommended.

INTRODUCTION

Column technology today has reached a very advanced stage of development. It is now possible to define the optimum column (in terms of length, radius and particle diameter of the packing) that will separate a given mixture, employing a particular phase system, in the minimum time and with the minimum mobile phase consumption. Such optimum columns can be defined for separations carried out by both gas chromatography (GC) and liquid chromatography (LC) and for both packed and capillary columns[1,2]. A protocol for column design has been established[1] and the constraints imposed upon the separation by the instrument specification limits in terms of analysis time, sample size and solvent consumption are now recognized and understood. Furthermore, it is now possible to predict, with some accuracy, future improvements in high-speed separations and in the resolution of highly complex mixtures, that will be achievable with conventional chromatographic systems which employ pressure induced mobile phase flow-rates.

Modern column technology has evolved over a period of more than thirty years, but it is only over the last five years that the various aspects of column theory and practice have been brought together in a rational form to provide a sound basis for column design. In this paper, the development of column theory will be traced over the last thirty years, together with the progress in column technology that has resulted. In particular, its impact on column design in LC will be discussed at each stage. Finally, the future practical limits of performance that can be expected from packed columns employing pressure-induced flow-rates will be considered.

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THE DEVELOPMENT OF CHROMATOGRAPHIC THEORY

The first major contribution to the theory of chromatography was the plate theory which was developed by Martin and Synge[3] and extended by Keulemans[4]. In its original form, the plate theory explained retention in the terms of distribution coefficient and allowed the variance of a peak to the estimated as inversely proportional to the number of theoretical plates in the column. Consequently, the plate theory *per se* provided an equation that allowed the efficiency of a column to be calculated *after* it had been constructed, but this did not help much in column design. However, in 1959 Purnell[5] used the plate theory to develop an equation that allowed the number of plates required to effect any given separation to be calculated from the separation ratio of the closest eluted pair and the capacity factor of the first eluted peak of the pair. The equation of Purnell is given as follows

$$n = 16(1 + k')^2/k'^2(\alpha - 1)^2 \quad (1)$$

where n is the number of theoretical plates required, α is the separation ratio of the closest eluted pair and k' is the capacity factor of the first member of the closest eluted pair. (In this respect eqn. 1 differs slightly from that of Purnell, in that it employs the k' value of the first eluted peak as opposed to that of the second eluted peak.)

The relationship between the required number of theoretical plates to effect a separation as a function of the separation ratio and the k' value of the first eluted peak is shown in Fig. 1.

Purnell's equation demonstrated for the first time the need for extremely high efficiencies to separate solute pairs having *small* separation ratios eluted at low k' values. In Fig. 1, it is seen that to resolve a pair of solutes having a separation ratio of 1.01 and eluted at a k' of 1 requires more than half a million theoretical plates. The effect of k' is also a little surprising. The efficiencies required to separate a solute pair having a separation ratio of 1.06 and eluted at a k' of 5 is only one third of that required at a k' of unity. As a consequence, the need for high efficiencies to separate solute pairs eluted at low k' values encourages the choice of a solvent (or solvent mixture) that will provide greater retention.

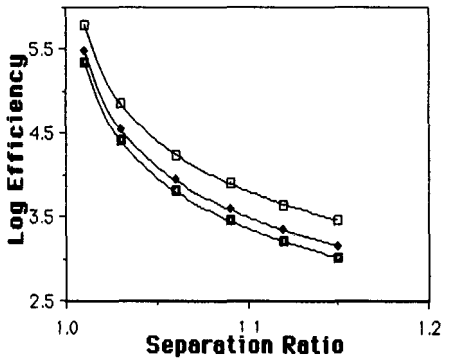


Fig. 1. Graphs of efficiency against separation ratio for different values of k': □, $k' = 1$; ◆, $k' = 2.5$; ⅜, $k' = 5$.

Eqn. 1 was of primary importance, as it allowed the efficiency needed to achieve any given separation to be calculated, but this was only a beginning. Before proceeding further, it is necessary to introduce the concept of the *reduced chromatogram.* Any chromatogram of a complex mixture of solutes can be reduced to a simple separation that will concisely describe the chromatographic problem. An example of a reduced chromatogram is shown in Fig. 2.

The reduced chromatogram consists of four peaks, first the dead volume peak, then a pair of peaks that are the two eluted closest together and thus the most difficult to separate. This pair of peaks is termed the *critical pair.* The column must be designed to separate the critical pair and, if this is satisfactorily achieved, then all the other peaks, which by definition are less difficult to separate, will also be resolved. The fourth peak in the reduced chromatogram is the last peak in the mixture which must be eluted to complete the analysis.

However, it shoukd be pointed out that a given column, operated at a given flow-rate, can exhibit a range of efficiencies depending on the nature of the solute that is chosen to measure it[6]. (This dependence of column efficiency on solute type will be discussed later.) Consequently, under exceptional circumstances, the predicted conditions for the separation of the critical pair may *not* be suitable for another pair and complete resolution of all solutes may not be achieved. This could occur if the separation ratio of another solute pair, although *larger*, is very *close* to that of the critical pair but contains solutes, for example, of very different molecular weight. However, the probability of this situation arising is extremely remote and will not be considered in this review. Thus, from the reduced chromatogram and eqn. 1 the number of theoretical plates required to separate any given mixture can be calculated.

The next development in the theory of chromatography that was essential for column design was the rate theory, first introduced by Van Deemter in 1956. Van Deemter *et al.*[7] put forward an equation relating the variance per unit length of a column (which can be shown to be numerically equivalent to the height of the theoretical plate) to the linear velocity of the mobile phase and physical properties of the solute, mobile phase and column.

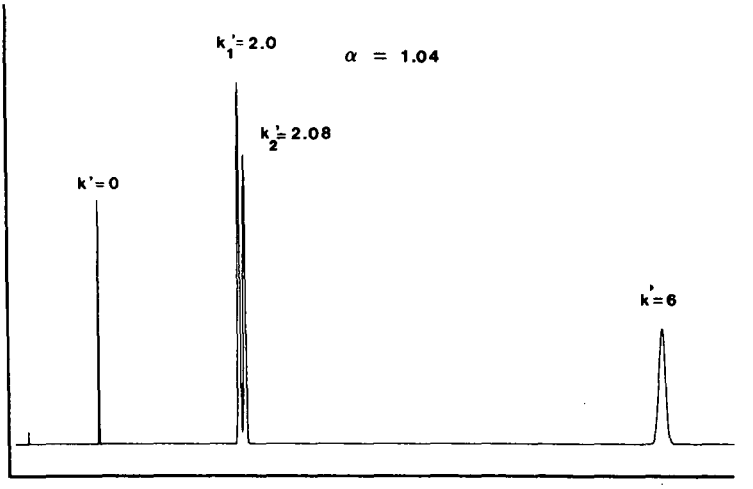


Fig. 2. The reduced chromatogram. Peaks: (1) $k'_0 = 0$; (2) $k'_1 = 2.0$; (3) $k'_2 = 2.08$; (4) $k'_3 = 6.0$. $\alpha = 1.04$.

Their equation took the following form.

$$H = A + B/u + Cu \tag{2}$$

where H is the variance per unit length of the column; A, B and C are constants for a particular chromatographic system, and u is the linear velocity of the mobile phase.

For a given column, a given mobile phase and a given solute, the expressions for the constants A, B and C are as follows:

$$A = 2\gamma d_p$$

where γ is a packing constant and d_p is the particle diameter of the packing.

$$B = 2\lambda D_M$$

where D_M is the diffusivity of the solute in the mobile phase and λ is a packing constant.

$$C = f_1(k')d_p^2/D_M + f_2(k')d_f^2/D_S$$

where d_f is the film thickness of the stationary phase and D_S is the diffusivity of the solute in the stationary phase.

Thus

$$H = 2\gamma d_p + 2\lambda D_M/u + f_1(k')d_p^2u/D_M + f_2(k')d_f^2u/D_S \tag{3}$$

Since the work of Van Deemter, a number of equations have been developed that purport to describe the variance per unit length of a column as a function of the linear mobile phase velocity[8–11]. However, each was carefully examined by Katz *et al.*[12], and it was concluded that the original equation of Van Deemter was as good as any, and better than most, for the precise prediction of the variance per unit length of a column particularly for linear velocities in the neighbourhood of the *optimum velocity*. Katz *et al.*[12] also showed that the resistance to mass transfer in the stationary phase contributed very little to the variance of the eluted peak, due to the very small value for d_f (the surface of the silica or bonded silica constituting the stationary phase). It was also established that $f_1(k')$ was very similar to the value derived for capillary columns, *viz.*

$$f_1(k') = (0.37 + 4.69k' + 4.04k'^2)/24(1 + k')^2 \tag{4}$$

Consequently eqn. 3 becomes

$$H = 2\gamma d_p + 2\lambda D_M/u + f_1(k')d_p^2u/D_M$$

or

$$H = 2\gamma d_p + 2\lambda D_M/u + (0.37 + 4.69k' + 4.04k'^2)d_p^2u/24(1 + k')^2D_M \tag{5}$$

An height equivalent to a theoretical plate (HETP) curve drawn from experimental data fitted to the Van Deemter equation, is shown in Fig. 3. It is seen that the fit is excellent. The Van Deemter equation can also provide a value for the optimum mobile phase velocity that must be employed with any given column. The optimum velocity will give the minimum variance per unit length and thus the maximum column efficiency. It was suggested by Knox and Saleen[13] and subsequently confirmed by Katz *et al.*[1] that the optimum velocity is that velocity which must be employed with the optimized column of minimum length to provide the minimum analysis time. To obtain the optimum velocity. Eqn. 4 is differentiated with respect to u and equated to zero. Thus, by solving for u it is seen that

$$u_{opt} = (B/C)^{0.5}$$

or

$$u_{opt} = \{2\lambda D_M/[f_1(k')d_p^2/D_M]\}^{0.5} \quad (6)$$

The minimum plate height, which is obtained at the optimum velocity, can be determined by substituting for u_{opt} in eqn. 2 from eqn. 6

$$H_{min} = A + 2(BC)^{0.5}$$

$$= 2\gamma d_p + 2[2\lambda D_M(0.37 + 4.69k' + 4.04k'^2)d_p^2/24(1 + k')^2 D_M]^{0.5} \quad (7)$$

Now, as the variance per unit length of the column is equal to the ratio of the column length (l), to the efficiency then, eqn. 7 can also provide an expression for the column length.

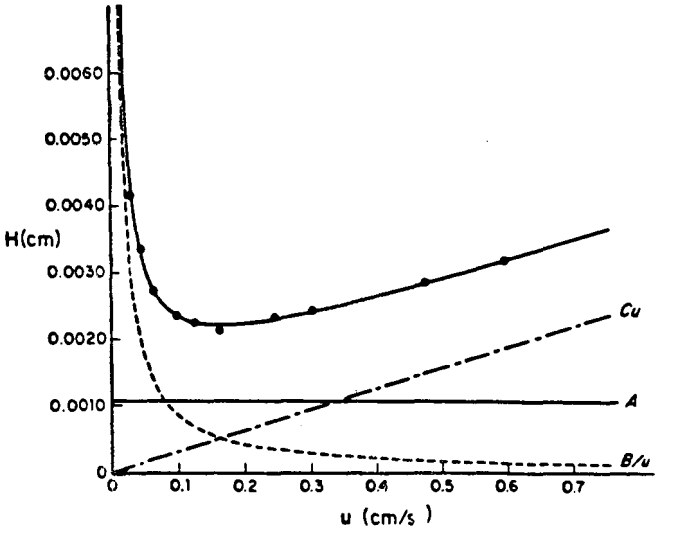


Fig. 3. Graph of HETP against linear mobile phase velocity (points curve fitted to the Van Deemter equation).

$$l = nH_{min} = n[A + 2(BC)^{0.5}]$$

$$= n\{2\gamma d_p + 2[2\lambda D_M(0.37 + 4.69k' + 4.04k'^2)d_p^2/24(1 + k')^2 D_M]\}^{0.5} \quad (8)$$

Eqn. 8 provides a value for the length of the column that is necessary to achieve the separation of the critical pair in the reduced chromatogram. However, this equation still contains one *undefined* variable, the particle diameter, d_p, the rest (*i.e.* γ, λ, D_M and k') being determined by the choice of the phase system and the quality of the packing. Consequently, it is now necessary to obtain an expression for the optimum particle diameter to be used in the packing.

Now, the particle size of the packing controls not only the value of H but also the permeability of the column. According to D'Arcy's law,

$$u = P\psi d_p^2/\eta l \quad (9)$$

where ψ is D'Arcy's constant for a packed bed, η is the viscosity of the mobile phase and P is the applied pressure, or, when the optimum mobile phase velocity is employed

$$u_{opt} = P\psi d_p^2/\eta l$$

or

$$l = P\psi d_p^2/\eta u_{opt} \quad (10)$$

However, from eqn. 8 it is also seen that:

$$l = nH_{min}$$

Equating expressions 8 and 10

$$nH_{min} = P\psi d_p^2/\eta u_{opt} \quad (11)$$

Substituting for H_{min} and u_{opt} from eqns. 6 and 7, respectively

$$n[A + 2(BC)^{0.5}] = P\psi d_p^2/\eta(B/C)^{0.5} \quad (12)$$

Solving for the particle diameter, d_p

$$d_p = (2\eta n D_M/\psi P\{\lambda[2\gamma/f(k')]^{0.5} + \gamma\})^{0.5} = d_{opt} \quad (13)$$

Thus, the particle diameter of the packing can be determined. It should be emphasized that this is the *optimum* particle diameter which will give the *minimum* analysis time.

It is now a simple substitution procedure to obtain an equation for the minimum analysis time (t), which is given by

$$t = (1 + k_2')l/u_{opt} = (1 + k_2')nH_{min}/u_{opt} \quad (14)$$

or

$$t = (1 + k'_2)n[A + 2(BC)^{0.5}]/(B/C)^{0.5} = (1 + k'_2)n[A(C/B)^{0.5} + 2C] \quad (15)$$

$$t = (1 + k'_2)n\{2\gamma d_{opt}[(0.37 + 4.69k' + 4.04k'^2)^2_{opt}/24(1 + k')^2 D_M^2 2\lambda]^{0.5} + \\ + 2[(0.37 + 4.69k' + 4.04k'_2)d^2_{opt}/24(1 + k')^2 D_M]\} \quad (16)$$

where r is the column radius and σ_A is the extra column dispersion in milliliters resulting from dispersive processes taking place *external to the column* in, *e.g.*, the sample valve, connecting tubes, detector cell etc. It is seen that the column radius depends only on the extra column dispersion of the chromatograph, the separation ratio of the critical pair, and the optimum particle diameter of the packing.

In summary the pertinent equations for column design are as follows:

Column efficiency $(n) = 16(1 + k')^2/k'^2(\alpha - 1)^2$

Particle diameter $(d_{opt}) = (2\eta n D_M/\psi P\{2\lambda[2\gamma/f(k')]^{0.5} + 2\gamma\})^{0.5}$

Optimum velocity $(u_{opt}) = \{2\lambda D_M/[f_1(k')d_p^2/D_M]\}^{0.5}$

Column length $(l) =$
$n\{2\gamma d_{opt} + 2[2\lambda D_M(0.37 + 4.69k' + 4.04k'^2)d^2_{opt}/24(1 + k')^2 D_M]\}^{0.5}$

Analytical time $(t) =$
$(1 + k'_2)n\{2\gamma d_{opt}[(0.37 + 4.69k' + 4.04k'^2)d^2_{opt}/24(1 + k')^2 D_M^2 2\lambda]^{0.5} +$
$+ 2[(0.37 + 4.69k' + 4.04k'^2)d^2_{opt}/24(1 + k')^2 D_M]\}$

Column diameter $(r) = [0.09\sigma_A(\alpha - 1)/d_{opt}]^{0.5}$

DISCUSSION

The equations given above demonstrate that there is one *unique* column that will resolve a given mixture in the minimum time and this column must be packed with particles of optimum diameter and operated at the optimum mobile phase velocity. As a result, there appears to be some conflict with traditional ideas on the subject which have usually assumed that for fast separations, velocities above the optimum should be employed and for high resolution and high efficiencies particle diameters should be made as small as possible. These misconceptions have arisen partly as a result of disregarding the fact that there is a limited inlet pressure available from the pump and partly from attempting to obtain fast separations from columns of fixed length. As a consequence of limited inlet pressure the particle diameter cannot be beyond that which will permit the optimum velocity to be realized. If higher efficiencies are required, the column must be made longer, and to achieve this, the column permeability must be increased by making the particle diameter larger.Velocities higher or lower than the optimum would increase the HETP and thus the required resolution would not be obtained.

However, if, for some reason, the length of the column cannot be changed then, for samples where the separation ratio of the critical pair is relatively high and the column has an efficiency in excess of that required, very fast separations can be achieved by operating at very high linear velocities. However, it must be emphasized that under these circumstances, although the separation will be fast, the analysis time will *not* be the minimum. The separation would be made even faster by reducing the particle size of the packing and employing a shorter column that could now operate at the optimum velocity. Unfortunately, as will be discussed later, the optimum particle size for a very simple separation may be smaller than the minimum available or below that which can be packed with known techniques. Under such circumstances, non-optimized columns with excess efficiency, operated at high velocities may, be the only way to reduce the analysis time to the required level.

The design equations will now be used to demonstrate the unique properties of optimized columns. For the most part, the main variable that will be employed will be the separation ratio of the critical pair, as this will demonstrate how the column properties vary with the difficulty of separation. In the examples given, the following values for the other pertinent variables will be assumed: packing constant (λ), 0.5; packing constant (γ), 0.6; diffusivity of the solute in the mobile phase (D_M), $3.5 \cdot 10^{-5}$ cm^2/s; mobile phase viscosity(η), 0.023 poises; D'Arcy constant (ψ), 35; capacity factor of the first of the critical pair (k'_1), 2.5; and capacity factor of the last eluted peak (k'_2), 5.0.

The values for the packing constants of 0.5 and 0.6 for λ and γ, respectively, are those predicted from theory by Giddings[15] and generally attainable by modern packing procedures. The value taken for the diffusivity of the solute in the mobile phase is for benzyl acetate in a mixture consisting of 5% (w/w) ethyl acetate in *n*-heptane, typical for many solute–solvent systems. The viscosity value taken is for the same solvent mixture. The D'Arcy constant was taken from measurements made on a number of columns packed with particles of different diameter by Katz *et al.*[1]. The k' values taken are also fairly typical for many routine chromatography analyses.

Employing eqn. 16 the analysis times were calculated for the resolution of three samples, the critical pairs having separation ratios of 1.02, 1.04 and 1.06 respectively. The results, plotted as curves relating analysis time to particle diameter are shown in Fig. 4. Included are curves relating the optimum particle diameter (calculated for three different inlet pressures) to the separation ratio of the critical pair. The other curves were calculated for an inlet pressure of 3000 p.s.i. Examination of Fig. 4 shows that there is, indeed, an optimum particle diameter that will provide the minimum analysis time and this optimum increases in magnitude with the difficulty of the separation. This again appears to be in conflict with accepted principles. However, it is clear that the more difficult separations must be accomplished with particles of larger diameter to provide adequate column permeability and, thus, permit the use of the necessary longer columns.

The more simple the separation, the more critical becomes the need to employ the optimum diameter if the minimum analysis time is to be achieved. Furthermore, it is seen that, if the particles are too small, the column will be operated below its optimum velocity, due to inadequate inlet pressure. Consequently, the variance per unit length will be increased as a result of the dominance of the longitudinal diffusion term in the Van Deemter equation, and a longer column will be necessary in order to

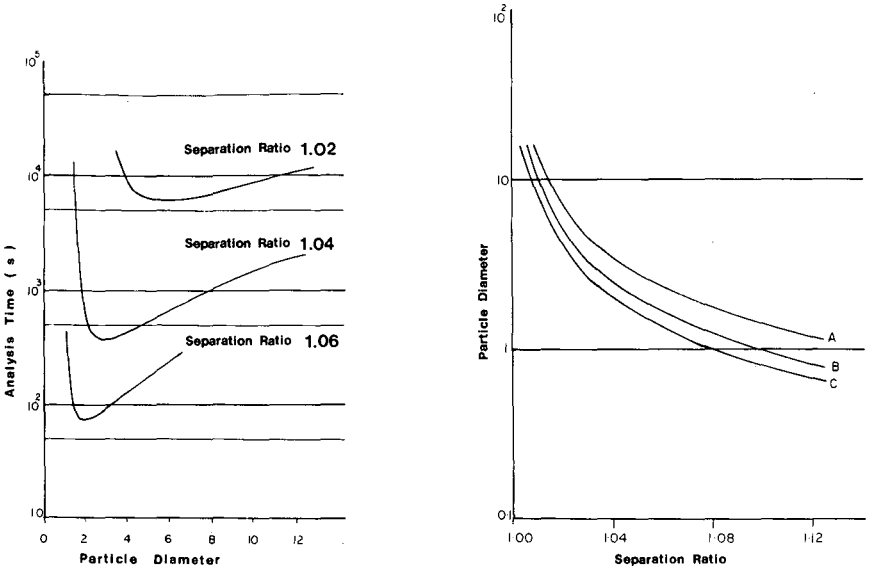


Fig. 4. (1) Graphs of analysis time against particle diameter (μm) for the separation of different solute pairs having different separation ratios. (2) Graphs of optimum particle diameter (μm) for minimum analysis time against separation ratio. Key: A = 2000 p.s.i.; B = 4000 p.s.i.; C = 6000 p.s.i.

attain the necessary efficiency. In a similar manner, if the particles are too large the dispersion will be greater at the optimum velocity due to the increased magnitude of the resistance to the mass transfer term in the Van Deemter equation. Consequently, the column must again be made longer to provide adequate efficiency and, as a result, the analysis time will also be extended.

It is seen from the second graph that, over the range of separation ratios chosen, the magnitude of the optimum particle diameter extends from *ca.* 0.8 μm to about 20 $\mu\mu$. It is also seen that, providing the inlet pressure available is above 2000 p.s.i., the effect of pressure on analysis time is not nearly as significant as might be expected. At present, particles of less than about 2 μm are not readily available and are fairly difficult to pack employing the usual slurry methods of packing.

It is also interesting to determine how the column length and analysis time for optimum columns change with the separation ratio of the critical pair. Employing 8 and 16, curves relating optimum column length and analysis time to the separation ratio of the critical pair were constructed and are shown in Fig. 5.

Fig. 5 shows that the analysis time can range from 2–3 s to *ca.* 2.8 h for separation ratios of 1.12 (a very simple separation) to 1.03 (a moderately difficult separation). An analysis time of 2.8 h appear long, but if the critical pair has a separation ratio of 1.03 and the maximum inlet pressure available is 6000 p.s.i. then this must be tolerated as no other column will provide a faster analysis.

Long analysis times appear as anathema to most chromatographers and many seem to think that by some clever design of column all mixtures, however complex, can be separated in a few minutes. Nothing could be farther from the truth. The cost of a chromatographic separation is paid for in two "currencies", time and pressure. This

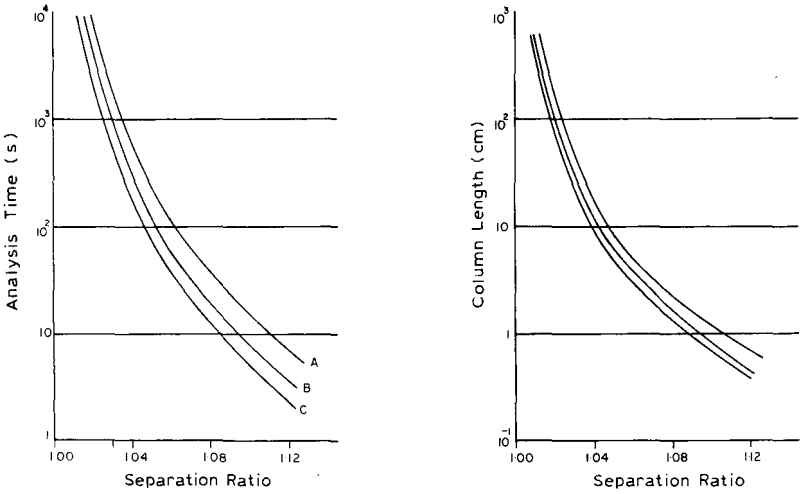


Fig. 5. (1) Graphs of analysis time obtained by the use of optimum diameter particles against separation ratio. (2) Graphs of column length against separation ratio for columns packed with particles of optimum diameter. Key: A = 2000 p.s.i.; B = 4000 p.s.i.; C = 6000 p.s.i.

was clearly stated by Golay[16] in 1960 when he introduced the Performance Index and, as the inlet pressure of any chromatographic system has a practical limit, time is the only variable left to expend to ensure resolution. *The impatient chromatographer must seek a simple sample.*

The relationship between the optimum column radius and the separation ratio of the critical pair is given by eqn. 16 and is graphically represented in Fig. 6. The standard deviation (S.D.) resulting solely from extra column dispersion was taken to be $2.5 \cdot 10^{-3}$ ml.

Fig. 6 shows that the optimum column radius increases linearly with the separation ratio of the critical pair. The optimum radius (which will depend on the extra column dispersion of the apparatus) ranges from about 5 mm for a separation ratio of 1.12–0.5 mm for a difficult separation of 1.02. Thus, complex mixtures that are *difficult* to separate would be carried out on long, thin columns and *simple* separations carried out on short, wide columns.

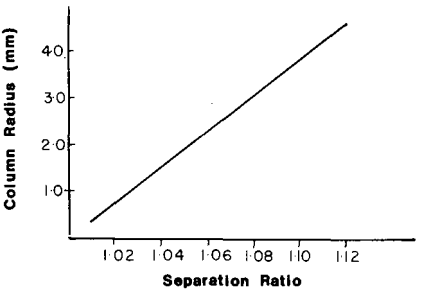


Fig. 6. Graph of optimum column radius against the separation ratio of the critical pair.

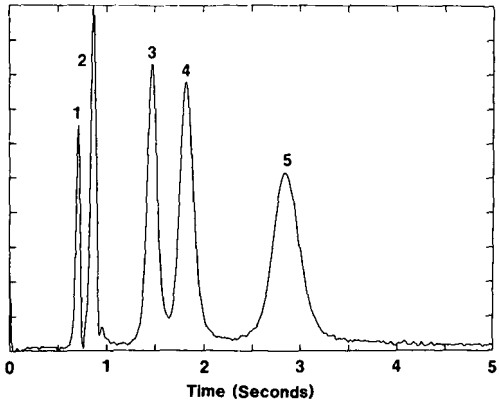
EXAMPLES OF EXCEPTIONAL COLUMN PERFORMANCE

Very high-speed separations and high-resolution columns can only be realized by designing the column to be optimum, or near optimum for the particular analysis to be carried out. An example of a high-speed separation taken from the work of Katz and Scott[17] and carried out on a near optimum column is shown in Fig. 7.

It is seen that the separation of five solutes is accomplished in less than 4 s. The column used was 2.5 cm long and this was the shortest that could be packed efficiently by the equipment available at that time. The particle diameter was 3 μm and this was also the smallest diameter packing available that had a sufficiently narrow particle size distribution for quality packing. The column diameter of 2.6 mm was appropriate for the extra column dispersion present in the instrument. The separation ratio of the critical pair (peaks 3 and 4) had a separation ratio of 1.5 and thus required particles of less than 1 μm in diameter for optimum performance. This would also require a column length of less than 1 cm. As a consequence the actual column used would have had an excess of efficiency for the separation required, if operated at the optimum velocity. Thus, to achieve a rapid separation, the linear velocity was increased to 3.3 cm/s (the rationale for this methods of operation has been previously discussed). This held the speed record in LC for a number of years.

Separations such as this, although demonstrating one aspect of the efficacy of the technique, are really part of chromatography 'show biz' as there are very few application where analyses of this speed are required. Perhaps such speeds might find use in work associated with fast reaction kinetics, or continuous toxicity monitoring, but in most analytical laboratories, results produced at this speed would provide an embarrassing problem of accurate interpretation and sensible subsequent action.

Very fast separations in LC can only be achieved for simple mixtures where there is little chromatographic challenge, that is to say, for mixtures were the critical pairs have large separation ratios. For example, the first pair of peaks in Fig. 7 has a separation ratio of *ca.* 5, and thus, even at the low k' value for the second peak of 0.2 very few theoretical plates are required to effect a separation. In Fig. 8, the first two



	k'	N	N/s
1 p-xylene	0	1100	1450
2 Anisole	0.2	1080	1200
3 Nitrobenzene	1.0	840	560
4 Acetophenone	1.5	800	430
5 Dipropyl Phthalate	2.9	450	160

Fig. 7. High-speed chromatography. Packing, Hypersil 3 μm; column, 2.5 cm × 0.26 cm I.D.; Linearly Velocity 3.3 cm/s. N = column efficiency.

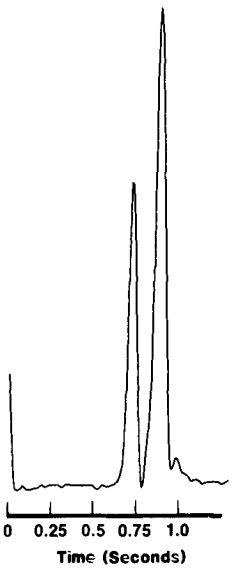


Fig. 8. Subsecond separation on a 2.5-cm column. Peaks: 1 = *p*-xylene, 2 = anisole.

peaks are shown as an isolated chromatogram and includes some analytical data taken from the same woerk of Katz and Scott[17] and are given in Table I.

It is seen that the separation is complete in about 900 m and the first peak is eluted in about 750 ms. Although the separation was very fast, demonstrating the limits of chromatographic speed at that time (1982), it would have very little practical use for the same reasons as those given for the parent chromatogram. It is interesting to note, however, that retention time and area precision of measurement would be quite satisfactory for many analyses, if such speeds were ever called for.

At the other extreme, very-high-efficiency columns, capable of resolving very difficult mixtures, will exhibit analysis times of many hours. In Fig. 9 a chromatogram from a column providing 160 000 theoretical plates shows the separation of a sample of cinnamon bark oil.

The column used was 10 m × 1 mm I.D. It was packed with particles of 20 μm in diameter, which is close to the optimum particle size for this length of column. The flow-rate employed was 38 μl/min, which was significantly above the optimum. At the optimum flow-rate the column gave 250 000 theoretical plates which is the efficiency to

TABLE I

CHROMATOGRAPHIC DETAILS FOR THE SEPARATION SHOWN IN FIG. 8.

	Normalized peak area			*Retention time (ms)*		
	Mean (6 runs)	*σ (S.D.)*	*%σ (R.S.D.)*	*Mean (6 runs)*	*σ (S.D.)*	*%σ (R.S.D.)*
p-Xylene	44.82	0.54	1.2	730	2.2	0.4
Anisole	55.2	0.55	1.0	891	5.9	0.7

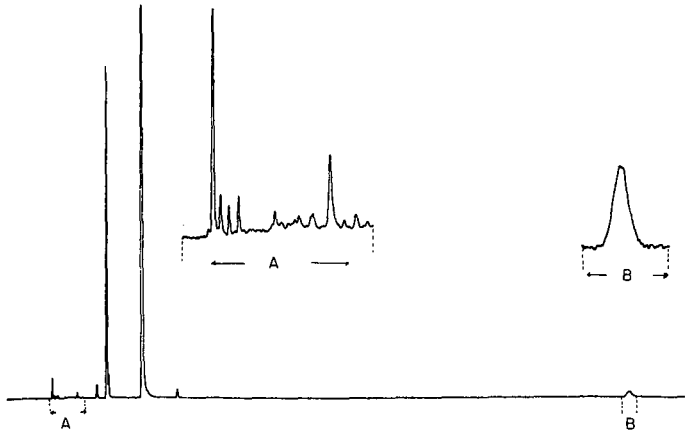


Fig. 9. Chromatogram of cinnamon bark oil. Column, 10 m × 1 mm I.D.; packing, Partisil 20; mobile phase, ethyl acetate–*n*-heptane (3:97, v/v); sample volume, 0.5 μl, flow-rate, 38 μl/min.

be expected from a 10-m column packed with 20-μm particles. The analysis time was over 52 h, but this was largely a result of the large k' value of the last eluted peak. It is interesting to note from the enlargement of the last peak, that even when eluted at a k' of about 50, the peak profile is still symmetrical. The enlargement of the early part of the chromatogram emphasizes the high resolution that can be obtained from the column. The last two, small peaks, shown in the enlargement are very well resolved and have a separation ratio of only about 1.07, the first solute being eluted at a k' value of less than unity.

Thus, modern chromatographic theory provides the equations necessary to predict the optimum column design and operating conditions necessary to resolve any given sample mixture. Unfortunately, due to the practical constraints of the apparatus and the limited availability of certain packing materials, it is often not possible to fabricate the optimum column. Furthermore, even if the column is fabricated, the separation may take an impossible length of time to complete. It follows that it is necessary to know the practical limits of column dimensions and operating conditions and, equally important, how these limits restrict the range of sample complexity that can be satisfactorily handled by LC analyses.

THE COLUMN OF THE FUTURE

Future LC columns of the conventional type, employing pressure-induced flow-rates will, perhaps a little disappointingly, not differ greatly from those used at present. Analysis times may be reduced to a few milliseconds for the separation of solutes having relatively large separation ratios, by employing small columns packed with the smallest available particles and operated at very high mobile phase velocities. However, the areas of application of such columns will, indeed, be very limited. At the other end of the scale, 1 or 2 million theoretical plates are obtainable from long, thin columns, packed with particles of 20 or 30 μm in diameter, but these will involve analysis times extending over several days if real samples are to be analyzed.

In Table II the properties of a number of optimized columns, suitable for the

TABLE II

PROPERTIES OF SOME OPTIMIZED LC COLUMNS

Separation ratio	*Column efficiency*	*Particle diameter (μm)*	*Column length (cm)*	*Column radius (cm)*	*Analysis time*
1.001	$3.1 \cdot 10^7$	110	615 016	$3.5 \cdot 10^{-3}$	84 years
1.005	$1.3 \cdot 10^6$	22	7667	0.02	7.2 days
1.010	313 000	11	613	0.035	10.4 h
1.050	12 500	2.2	4.9	0.175	1 min
1.100	3140	1.1	0.6	0.35	3.8 s
1.200	784	0.5	>0.1	0.73	270 ms

separation of samples covering a wide range of difficulty, are given. These results were obtained by the use of the equations previously presented. The basic data used in the calculations are the same as those previously defined, except for pressure, which was taken as 3000 p.s.i.

The results shown in Table II clearly indicate the practical range of separations that are amenable to LC analysis. It is obviously that a column 6 km long with an analysis time of 84 years would only be useful in some "time dimension" other than our own. In fact, the column 76 m long, less than 1 mm in diameter with an analysis time of over 7 days is only just feasible. Furthermore, the problem would, indeed, have to be very important, if its construction and use were to be justified. The real practical column limit for high resolution starts with the 6-m column, just under 1 mm in diameter, packed with 11-μm particles and requiring an analysis time of about 10 h. This column would separate samples where the separation ratio for the critical pair was as low as 1.01. At the other extreme, a column less than 1 mm long, packed with 0.5-μm particles and providing separations in less than 300 ms is also not practical tp construct or operate, and the availability of closely graded particles, less that 1 μm in diameter is, at best, a speciality at this time. In fact, particles less than 2 μm in diameter are still somewhat of a novelty. As a consequence, the fastest practical column for the separation of relatively simple mixtures, is probably the 5-cm column, *ca.* 4 mm in diameter, packed with 2.2-μm particles, which would complete separations in about one min. In fact, a family of three or four columns, spanning the range of separation ratios between 1.01 and 1.10 would be the most practical for analytical purposes. However, before this column family is considered, there should be some discussion on the maximum inlet pressures to the column. In the calculations necessary to provide the data given in Table II, an inlet pressure of 3000 p.s.i. was assumed. Pumps are readily available that will provide pressures of 6000 or even 10 000 p.s.i. but, unfortunately, it is not the pump that controls the operating pressure of the column system. The sample valve is the component most liable to leakage at high pressures, particularly after prolonged use. Valves are also manufactured for use at 6000 p.s.i. and even more, but unfortunately, in continuous use, the life of such valves tend to be limited. Nevertheless, most commercially available valves will work for long periods at 3000 p.s.i. without leaking, and for this reason, this pressure was chosen as appropriate for the calculation. Finally, it must be said that columns a little outside the range

TABLE III

PRACTICAL FAMILY OF COLUMNS FOR GENERAL LC APPLICATIONS

	Separation ratio	*Column efficiency*	*Particle diameter (μm)*	*Column length (cm)*	*Analysis time (min)*
1	1.02	78 400	5.5	77	40
2	1.03	34 840	3.7	23	8
3	1.05	12 500	2.2	4.9	1

suggested could well be practical, if higher pressures could be used, long analysis time were tolerated, or smaller particles became readily available, together with satisfactory packing procedures for them. Higher pressures would result in considerable heat generation, so the column would need to be well thermostated with fluids of high heat capacity.

The following set of three columns is recommended to cover a practical range of LC separations (Table III). They can be packed with either silica gel or a bonded phase of choice.

The columns are not available as standard items, and both columns 1 and 2 would probably need to be packed in separate lengths and then joined. Column 1 could be packed in three 25-cm lengths and column 2 in two 12-cm lengths. Particle sizes close to those required for columns 1 and 2 are readily available and the 2.2-μm particles for column 3 are becoming available through certain manufacturers. Optimum column diameters are not given, as they will depend on the type of chromatograph with which the columns are to be associated. It is likely that a compromise value for the column diameter that might be satisfactory for the majority of instruments would be 2 mm. It should be emphasized, however, that the extra-column dispersion of the instrument should be known and the optimum column diameter should be calculated and used. Finally, it must be said that for routine analyses, where the same sample is analyzed many times over long periods, then it is well worth identifying the optimum column for the particular analysis, employing the equations given above, and have it custom-made. The result would be significant economic savings in both time and solvent consumption.

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CHROMSYMP. 1522

SUPERCRITICAL-FLUID CHROMATOGRAPHY–MASS SPECTROMETRY OF POLYCYCLIC AROMATIC HYDROCARBONS WITH A SIMPLE CAPILLARY DIRECT INTERFACE

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SUMMARY

Supercritical-fluid chromatography–mass spectrometry (SCF–MS) with carbon dioxide has been used to separate and identify polycyclic aromatic hydrocarbons (PAHs) and heteroatom-containing PAHs with molecular weights up to 532. The capillary direct interface allowed methane chemical ionization (CI) mass spectra to be obtained without modification of a commercially available quadrupole mass spectrometer, and a single instrument can be used for SFC–MS and gas chromatography–MS with a conversion between modes requiring *ca.* 20 min. The interface yields good chromatographic peak shapes, and full-scan spectra were obtained at the low ng level. With selected-ion monitoring, detection limits of *ca.* 25 pg were achieved. SFC–MS analysis of an extract from treated wood and the methane CI mass spectra of 29 standard PAHs and heteroatom-containing PAHs are reported.

INTRODUCTION

Capillary gas chromatography–mass spectrometry (GC–MS) is an extremely powerful technique for the analysis of samples containing polycyclic aromatic hydrocarbons (PAHs) and heteroatom-containing PAHs. However, the ability of conventional GC techniques to elute high-molecular-weight and more polar PAHs is limited by their low volatility. High-performance liquid chromatography (HPLC) has been used for the separation of higher-molecular-weight PAHs, but suffers from lower chromatographic resolution per unit time and, despite intensive attempts by several investigators, the routine coupling of HPLC with MS has proven to be difficult.

Capillary supercritical-fluid chromatography (SFC) is a rapidly developing method for the separation and identification of organic compounds that lack sufficient volatility or thermal stability to be separated by GC. The use of SFC to separate higher-molecular-weight PAHs has been demonstrated, and the coupling of SFC with MS, which has been reported by several investigators, has been the subject of recent reviews[1–9].

In general, the SFC interfaces that have been reported are based on modifications of the existing mass spectrometer interface and/or the ion source, which require

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dedication of the mass spectrometer to SFC–MS. For many laboratories, the limited availability of instrumentation and the need to perform conventional GC–MS analyses eliminates the possibility of committing a mass spectrometer to SFC–MS. In such cases, simple SFC–MS interfaces are needed which will allow a single instrument to be interconverted routinely between GC–MS and SFC–MS with a minimum of time. A recent preliminary report[10] has described a simple SFC–MS capillary direct interfacing method which allows a single quadrupole mass spectrometer to be used for GC–MS and SFC–MS with minimal conversion time. In the present report, the use of the simple capillary direct interface to obtain methane chemical ionization (CI) spectra of PAHs and heteratom-containing PAHs under SFC–MS conditions with carbon dioxide is reported.

EXPERIMENTAL

All SFC–MS analyses were performed with a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 5988 GC–MS system. The Model 5890 gas chromatograph that was supplied with the instrument was converted for SFC by installing a Valco (Houston, TX, U.S.A.) Model CI4W HPLC valve, equipped with a 0.5-μl sample loop, above one of the detector holes on the top of the gas chromatograph. (The detector holes are unused, since the interface to the mass spectrometer exits through the side of the GC oven.) The SFC column and the injector splitter assembly entered the oven through one of the detector holes, and were constructed as described by Peadon *et al.*[11]. A short piece of 10-μm I.D. fused-silica capillary tubing was used to control the split flow to obtain a split ratio of *ca.* 1:20.

The interfacing of the SFC column to the mass spectrometer was achieved by inserting the column through the transfer line until the restrictor tip of the column extended to *ca.* 1 mm of the end of the interface probe (*i.e.*, the SFC column was installed in a manner identical to that used for a capillary GC column). An integral "Guthrie" restrictor[12] was used to control column flow at a gas flow-rate of 1–3 ml/min, measured at 200 atm column pressure. No modifications of the commercially supplied GC–MS interface (such as the addition of heating elements at the column outlet) were made. The source temperature and the interface probe temperature were held at 270°C (the same as for the normal GC–MS mode of the instrument).

Since the GC injection ports remained in place, and since the mass spectrometer interface was not modified, converting the instrument from the SFC–MS to the GC–MS mode and back is achieved by simply installing the appropriate capillary column. Chromatographic columns can be changed in the Model 5988 GC–MS system without venting the source. This allows the conversion to be completed in *ca.* 20 min. The use of the integral restrictor for the SFC column also facilitated conversion, since the restrictor tip is phycically strong enough to allow removal and reinstallation of the SFC column without breaking.

SFC pressure programming was performed using a Lee Scientific (Salt Lake City, UT, U.S.A.) Model 501 pump. All separations were achieved with carbon dioxide as the carrier and a 10-m SB-phenyl-5 column (50 μm I.D., 0.5 μm film thickness), supplied by Lee Scientific.

Methane CI mass spectra (positive ion) were obtained with a source pressure (measured in the ion volume) of *ca.* 0.3 Torr when the SFC column pressure was 80

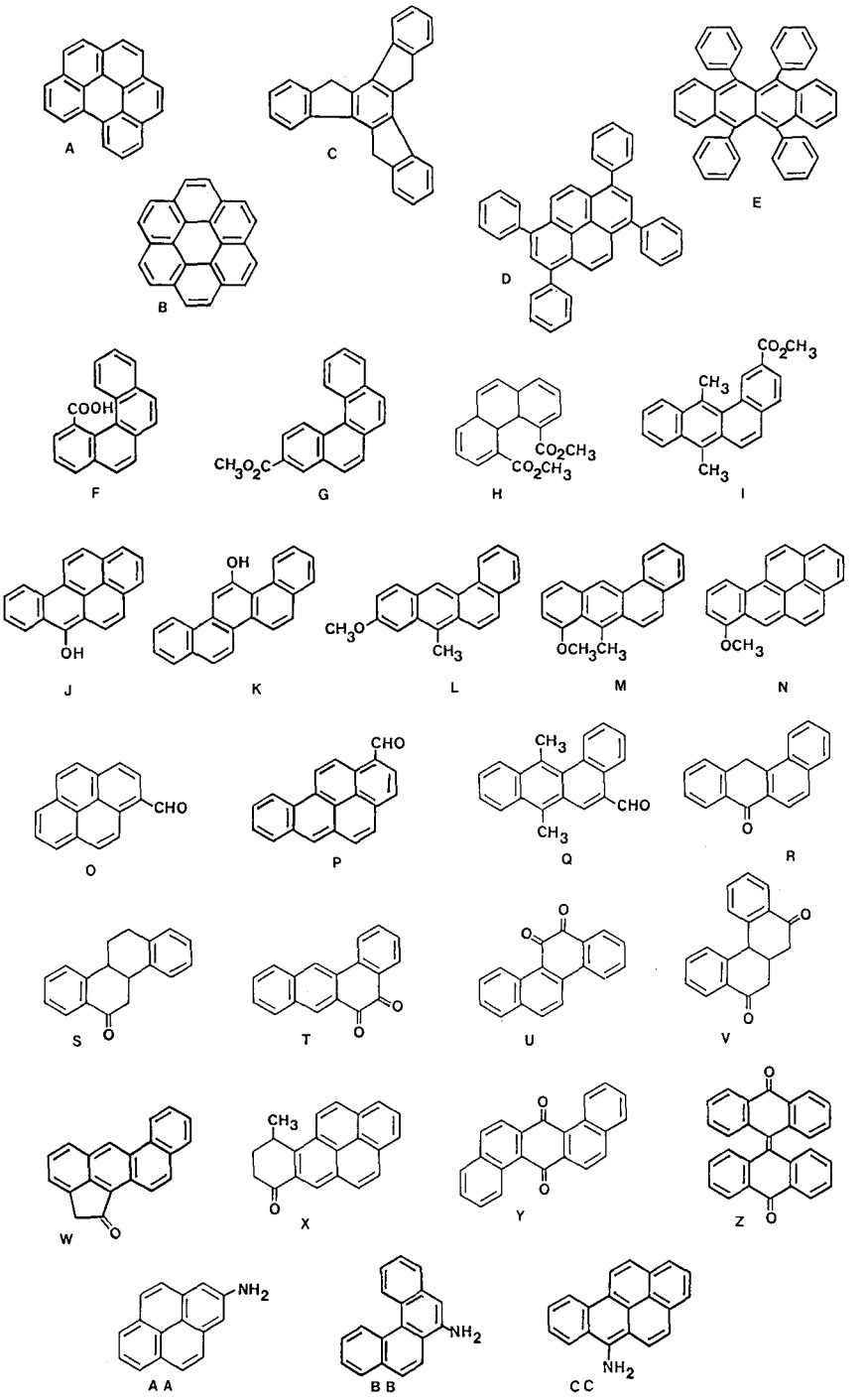

Fig. 1. Structures of the standard PAHs and functionalized PAHs. The letters under each structure correspond to the names and spectra given in Table I.

atm. (Source pressure increased to *ca.* 0.8 Torr during pressure programming up to 400 atm.) Tuning of the mass spectrometer source parameters was performed with polydimethylsiloxane, which was introduced using the direct insertion probe[13] and maximizing the intensity of the ions which occurred at 221, 369, 443, and 591 a.m.u. The ionizing voltage was typically 100 eV. Scan rate was *ca.* 450 a.m.u./s with a typical multiplier voltage of 2700. Standard PAHs and functionalized PAHs were obtained from Aldrich (Milwaukee, WI, U.S.A.) and dissolved in either chloroform or chlorobenzene to a concentration of *ca.* 1 mg/ml for SFC–MS analysis. The structures of the individual standards are shown in Fig. 1.

RESULTS AND DISCUSSION

During the initial development of the capillary direct interface, there was concern that the high pressure of carbon dioxide in the CI source during the SFC separation would produce intense background ions that could interfere with obtaining mass spectra in the lower mass range. Fig. 2 (top) shows the background ions in the spectrum between 50 and 1000 a.m.u. when the column pressure is 200 atm. Although an intense ion appears at $m/z = 59$ (presumably $[O=C-O-CH_3]^+$), only low-intensity background ions occur throughout the rest of the spectrum. The bottom half of Fig. 2 shows the magnitude of the background total ion current (TIC) when the lower limit of the scan range was raised from 50 a.m.u. to 65, 95, and 160 a.m.u. (with 1000 a.m.u. as the upper scan limit in each case). As shown in Fig. 2, the intensity of the TIC from the background ions decreased by a factor of *ca.* 2 for each increase in the lower scan limit. While the total intensity of background ions was fairly high when $m/z = 59$ was included in the scan range, beginning the scan above the mass of this ion gave an acceptable background. Since preliminary results showed that the PAHs and functionalized PAHs gave no significant low-mass fragments under the methane CI conditions, the remaining mass spectra reported in this study were obtained with a lower scan limit of 160 a.m.u. The resulting background was very low, and no

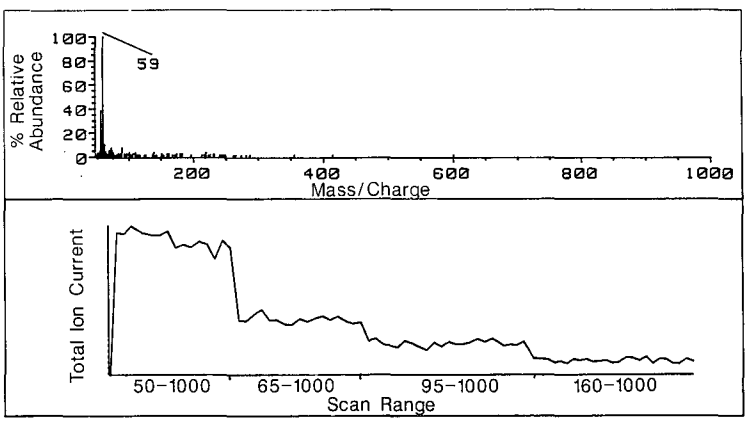

Fig. 2. Background ions in the scan range of 50 to 1000 a.m.u. (top) and the TIC of background ions at different scan ranges (bottom), obtained under SFC–MS conditions with methane chemical ionization. The column pressure was held at 200 atm.

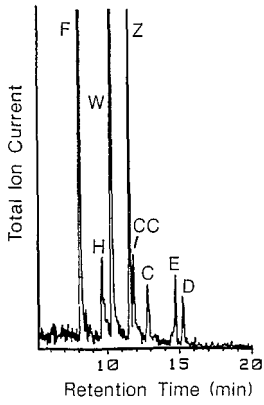

Fig. 3. TIC chromatogram of a mixture of PAHs and functionalized PAHs. The letters on the chromatogram refer to the structures shown in Fig. 1. SFC–MS conditions are given in the text.

background subtraction was used for any of the spectra or chromatograms shown throughout the rest of this report.

The use of the capillary-direct interface to obtain an SFC–MS TIC chromatogram of a mixture of PAHs, oxygenated PAHs, and an amino-PAH is shown in Fig. 3. Approximately 5 to 15 ng of each species were injected at 80 atm and an oven temperature of 125°C, followed by a pressure program of 20 atm/min to 400 atm. A coincident temperature program of 2°C/min to 200°C was also used since slightly better separation of the later-eluting species resulted than when pressure programming was performed at 125°C. The mass spectral scan range was 160 to 1000 a.m.u., and no attempt to reduce the baseline noise level (*i.e.*, background subtraction) was made.

As shown in Fig. 3, the capillary direct interface yielded acceptable chromatographic peak shapes for all of the PAHs, including an underivatized carboxylic acid (benzo[*a*]phenanthrene carboxylic acid, M = 272) and the highest-molecular-weight PAH available for this study (5,6,11,12-tetraphenylnaphthacene, M = 532). The chromatographic peak shapes shown in Fig. 3 also compare favorably to the peak shapes obtained when the same sample was analyzed under the same chromatographic conditions with a flame ionization detector (not shown).

Representative methane CI mass spectra obtained under the SFC–MS conditions described above are shown in Fig. 4, and the spectra for 29 standard species are summarized in Table I. Each of the spectra was generated with *ca* 50 ng of the test species, injected on-column. With only two exceptions (benzo[*c*]phenanthrene carboxylic acid and dimethyl-4,5-phenanthrenedicarboxylate), all of the standard compounds show the highest intensity ion (base peak) at the pseudomolecular ion (M + 1), formed from the addition of H^+. The adduct ion formed from the addition of $C_2H_5^+$ at M + 29 is apparent in all of the spectra. Most of the compounds also show an ion at M^+ which indicates that some charge exchange ionization (from the carbon dioxide) may be occurring under these conditions.

Many of the PAHs and functionalized PAHs show little fragmentation. This might be expected, since the electron impact spectra of such species also generally show little fragmentation. However, several of the compounds give useful fragmentation patterns. For example, each of the esters shows an ion at M − 31, corresponding to the

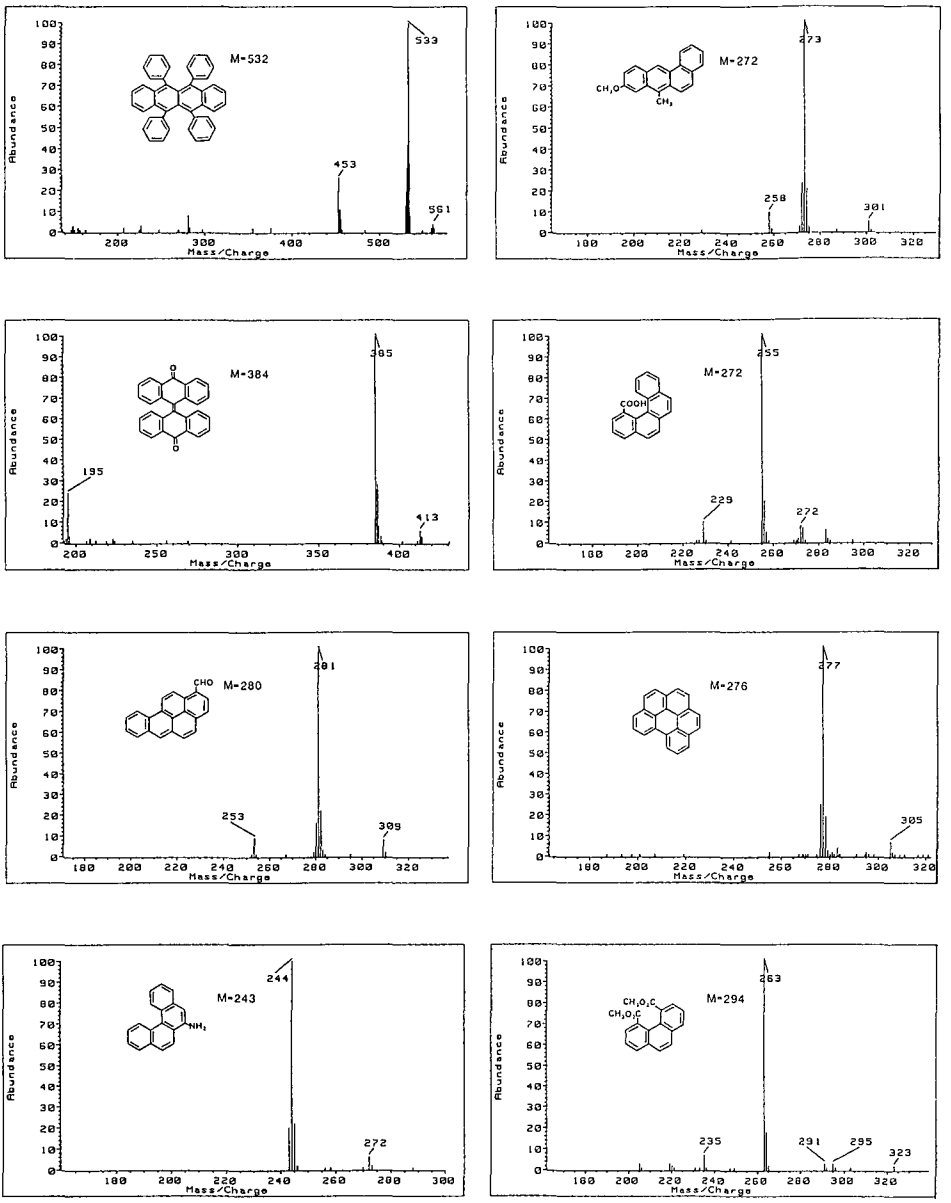

Fig. 4. Representative methane CI mass spectra obtained under SFC–MS conditions.

loss of methanol from the ionized parent. Benzo[*c*]phenanthrene carboxylic acid shows a base peak at $m/z = 255$ due to the loss of water from the ionized parent. This acid also shows significant M and M + 1 ions as well as an ion at $m/z = 229$, due to the loss of CO_2 from the ionized parent. The diester, dimethyl-4,5-phenanthrenedicarboxylate (Fig. 1H), shows a base peak at $m/z = 263$, due to the loss of methanol from the

TABLE I

SFC–MS METHANE CI MASS SPECTRA OF PAHs AND FUNCTIONALIZED PAHs

Species[a]		*Mol. wt.*	*Relative intensity (%)*[b]				
			M+1	*M+29*	*Other ions (mass, intensity)*[c]		
PAHs							
A	Benzo[*ghi*]perylene	276	100	7	276(25)		
B	Coronene	300	100	12	300(31)		
C	Truxene	342	100	8	342(18)	268(9)	269(8)
D	1,3,6,8-Tetraphenylpyrene	506	100	5	506(21)		
E	5,6,11,12-Tetraphenylnaphthacene	532	100	5	532(40)	531(30)	453(27)
					455(12)	530(10)	
O-PAHs							
Acids and esters							
F	Benzo[*c*]phenanthrene carboxylic acid	272	9	1	255(100)	229(11)	272(10)
G	Methylbenzo[*c*]phenanthrene-7-carboxylate	286	100	7	286(11)	255(14)	243(9)
H	Dimethyl-4,5-phenanthrene-dicarboxylate	294	4	2	263(100)	235(9)	291(5)
					295(5)		
I	Methyl-1,12-dimethylbenz-[*a*]anthracene-2-carboxylate	314	100	6	314(20)	283(6)	
Alcohols and ethers							
J	6-Hydroxybenzo[*a*]pyrene	268	100	8	268(12)		
K	3-Hydroxypicene	294	100	16	294(18)		
L	9-Methoxy-7-methylbenz-[*a*]anthracene	272	100	5	272(24)	258(11)	
M	8-Methoxy-7-methylbenz-[*a*]anthracene	272	100	5	272(25)	258(5)	
N	7-Methoxybenzo[*a*]pyrene	282	100	2	282(28)		
Aldehydes and ketones							
O	4-Pyrenecarboxaldehyde	230	100	9	203(16)		
P	1-Benzo[*a*]pyrenecarboxaldehyde	280	100	9	280(16)	253(9)	
Q	7,12-Dimethylbenz[*a*]anthracene-5-carboxaldehyde	284	100	6	284(10)	257(5)	
R	7,12-Dimethylbenz-[*a*]anthracene-7-one	244	100	5	244(21)	259(6)	
S	Hexahydrochrysene-6-one	248	100	7	207(15)		
T	5,6-Dihydrobenz[*a*]anthracene-5,6-dione	258	100	2	231(23)		
U	11,12-Dihydrochrysene-11,12-dione	258	100	2	231(14)		
V	Hexahydrobenzo[*c*]phenanthrene-5,8-dione	262	100	7			
W	Cholanthren-1-one	268	100	8	268(14)	241(12)	
X	7,8,9,10-Tetrahydro-10-methyl-benzo[*a*]pyrene-7-one	284	100	7	284(12)		
Y	7,14-Dihydrodibenz[*a,h*]-anthracene-7,14-dione	308	100	8			
Z	Bianthrone	384	100	9	195(26)		
NPAHs							
AA	2-Aminopyrene	217	100	8	217(15)		
BB	6-Aminobenzo[*c*]phenanthrene	243	100	7	243(24)		
CC	6-Aminobenzo[*a*]pyrene	267	100	2	267(45)		

[a] The letters refer to the structures shown in Fig. 1. Species names are those used by the supplier.

[b] Each ion with a relative intensity > 5% is reported. Spectra are corrected for ^{13}C contribution.

[c] The mass of the ion is followed by its relative intensity in parentheses.

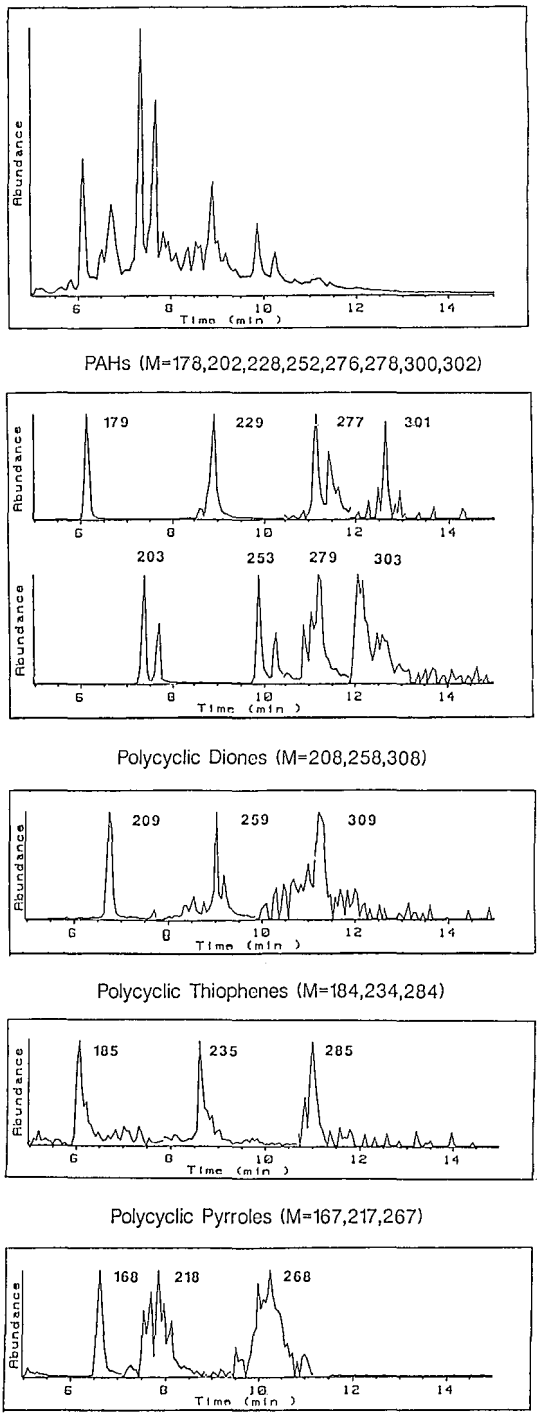

Fig. 5. SFC–MS analysis of a chloroform extract of a treated wooden utility pole. The TIC chromatogram (top) is shown along with the selected ion plots from the same SFC–MS analysis for the M + 1 ions of PAHs, polycyclic diones, polycyclic thiophenes, and polycyclic pyrroles. SFC–MS conditions are given in the text.

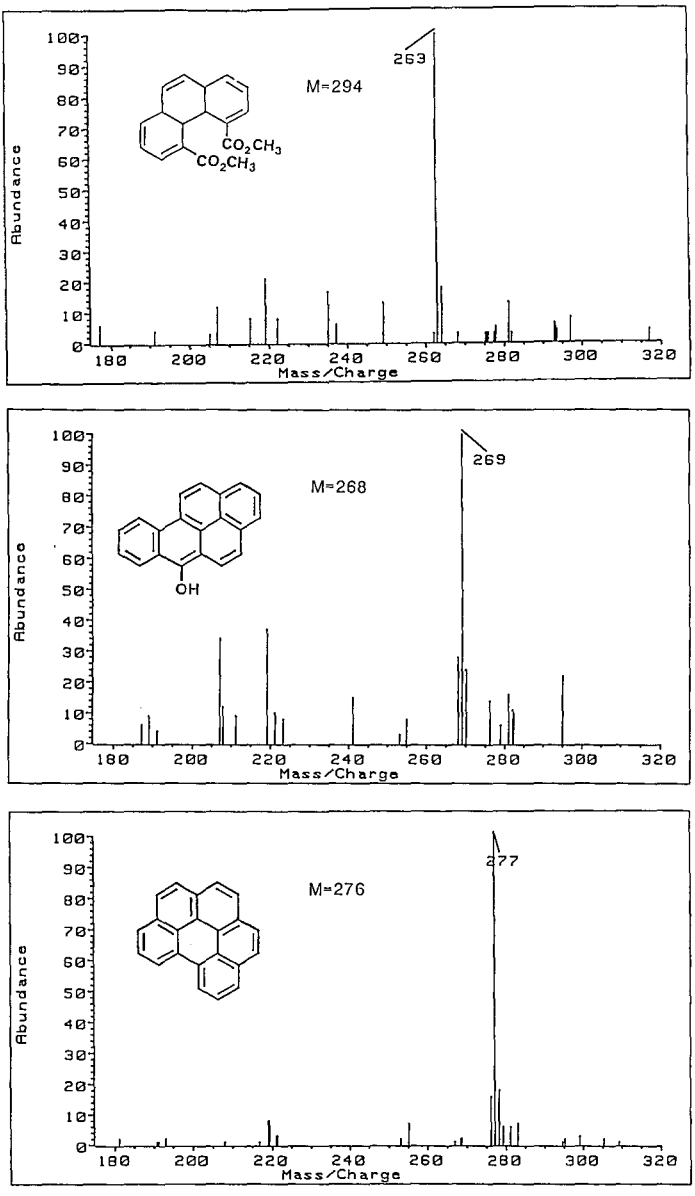

Fig. 6. SFC–MS full-scan (160–1000 a.m.u.) spectra, obtained with 2–3 ng each of dimethyl-4,5-phenanthrenedicarboxylate (H), 6-hydroxybenzo[*a*]pyrene (J), and benzo[*ghi*]perylene (A).

ionized parent. Significant ions were also observed at M+1, at the M+29 adduct (m/z = 323), as well as at m/z = 291, due to the loss of methanol from the adduct ion. Bianthrone (Fig. 1Z) shows a cleavage product ion at m/z = 195, while several of the other aldehydes and ketones show fragment ions at M−27, corresponding to the loss of CO from the ionized parent. While the fused-ring PAHs, coronene (Fig. 1B) and benzo[*ghi*]perylene (Fig. 1A), show no significant fragments (similar to their electron

impact spectra), both the 1,3,6,8-tetraphenylpyrene (Fig. 1D) and the 5,6,11,12-tetraphenylnaphthacene (Fig. 1E) show significant fragment ions.

The SFC–MS analysis of a complex PAH mixture is shown in Fig. 5 by the analysis of a chloroform extract from a treated wooden utility pole. The sample was injected at a pressure of 80 atm, followed by a pressure program of 20 atm/min to 400 atm. The oven temperature was programmed from 125°C to 200°C at 2°C/min. Because of the complexity of the sample, only the major PAHs (molecular weights ranging from 178 to 252) showed distinct chromatographic peaks in the TIC chromatogram, as shown in the top of Fig. 5. However, when the selected ion plots were constructed for the M + 1 ions of several PAHs and heteroatom-containing PAHs, several other species could be tentatively identified by using the same SFC–MS analysis. PAHs in the sample ranged from molecular weights of 178 to 302, polycyclic diones ranged from anthraquinone (M = 208) to dibenzoanthraquinone isomers (M = 308), polycyclic thiophenes ranged from dibenzothiophene (M = 184) to five-fused-ring thiophene isomers (M = 284), and polycyclic pyrroles ranged from carbazole (M = 167) to dibenzocarbazole isomers (M = 267).

The sensitivity achieved by use of the capillary direct interface in the full-scan mode (160–1000 a.m.u.) was investigated by injecting *ca.* 2–3 ng each of dimethyl-4,5-phenanthrenedicarboxylate, 6-hydroxybenzo[*a*]pyrene, and benzo[*ghi*]perylene. (The amount injected into the SFC column was calculated from the measured split ratio, the volume of the sample, and the concentration of the standard solution.) As shown in Fig. 6, the spectra (single scan) contained significant background ions that appear to be random system noise (no background subtraction has been used). However, the most intense ions for each species were easily recognizable and displayed ^{13}C isotope peaks with intensities that agree within *ca.* 3% of the calculated values, indicating that mass spectral data can be achieved at the ng level in the full-scan mode.

The sensitivity that could be achieved by using selected-ion monitoring (SIM) was also determined by injecting 22–35 pg of the same three species, and monitoring the pseudomolecular ions at M + 1 for 6-hydroxybenzo[*a*]pyrene and benzo[*ghi*]perylene and the ion at m/z = 263 for dimethyl-4,5-phenanthrenedicarboxylate. As shown

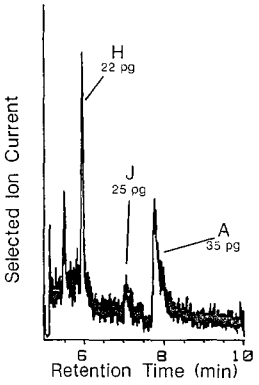

Fig. 7. Selected-ion current chromatogram of 22–35 pg each of dimethyl-4,5-phenanthrenedicarboxylate (H), 6-hydroxybenzo[*a*]pyrene (J), and benzo[*ghi*]perylene (A), obtained with SIM SFC–MS analysis. Chromatographic and SIM conditions are given in the text.

by the selected-ion current chromatogram in Fig. 7, each of the three species could be detected at the 22–35 pg level. The results shown in Figs. 6 and 7 demonstrate the ability of the simple capillary direct SFC–MS interface to yield full-scan and SIM sensitivities approaching those normally associated with conventional GC–MS analysis by the use of a quadrupole mass spectrometer operated in the positive-ion mode.

ACKNOWLEDGEMENTS

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SOME PRACTICAL ASPECTS OF COLUMN DESIGN FOR PACKED-COLUMN SUPERCRITICAL-FLUID CHROMATOGRAPHY

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SUMMARY

Different column configurations and column packings are evaluated for packed-column supercritical-fluid chromatography under pressure-programmed conditions. The best chromatographic performance was observed for columns of about 15–25 cm in length, packed to a moderate density with small-diameter particle packings of low surface area. Chemical and physical interactions of polar analytes with silanol groups were identified as a significant problem with available column packings that calls for the use of low-surface-area chemically bonded phases. Further deactivation by coating with a non-extractable, liquid organic salt is shown to modify the selectivity and activity of silica and chemically bonded silica phases. Recommendations are made for the selection of different colunm configurations for analytes of different kinds.

INTRODUCTION

The current revived interest in supercritical-fluid chromatography (SFC) is related to its ability to fill the gap between the capabilities of gas chromatography (GC) and liquid chromatography (LC) for the separation of middle-molecular-weight analytes, especially those of low thermal stability[1,2]. Supercritical fluids have viscosities somewhat similar to those of gases and diffusion coefficients approaching those of liquids. However, their most important characteristic is that their density is easily changed by changing pressure or temperature and in so doing the solubility characteristics of the analytes are dramatically changed. At temperatures above the critical temperature pressure or density programing permits a controlled change in chromatographic characteristics from GC-like at low pressure to LC-like at high pressures. For carbon dioxide, the most popular mobile phase in contemporary practice, the critical temperature is 31.05°C, thus enabling low temperatures to be used for the separation of thermally labile analytes. In terms of efficiency and speed of analysis, GC will always outperform SFC but for those samples of limited volatility and/or thermal stability SFC has the potential for faster analyses than LC.

Unlike the situation in GC and LC there is no kinetic model available to optimize packed-column SFC[2,3]. Also, in contemporary practice, most separations are performed with pressure or density programming, and it is conceivable that a dis-

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tinctly different threshold density exists for individual analytes before a retentive distribution mechanism begins to operate[4,5]. Since supercritical fluids are compressible, appreciable density drops may exist along the column, dependent on the column permeability and length. The column pressure drop (density gradient) can cause peak broadening due to solubility differences along the column[6–14]. Pressure programing produces significant velocity and viscosity gradients along the column[15–17]. This situation is further complicated in practice when pressure regulation is obtained by using a flow restrictor that results in a complex variation in linear velocity with pressure[18]. Pressure programing can also cause significant peak compression, leading to an increase in the quality of the separation. This can result from different mechanisms, for example, from the imbalance of mobile phase mass flow-rate in and out of the column under programed conditions or from the continuous precipitation and dissolution of the analyte along the column due to the existence of a column pressure drop, and the dependence of analyte solubility on density (pressure) and, therefore, migration rate. In both cases, the net result is that the tailing edge of the analyte peak moves faster than the leading edge thus reducing the natural zone broadening.

Several complex factors influence zone broadening in packed-column SFC, and the situation is much more difficult to model than GC or LC. Simply borrowing established models from GC or LC can be expected to represent only crude approximations. This led us to question the reasoning behind using columns optimized for LC in SFC. In the absence of a theoretical model we will take a phenomenological approach to column design under pressure-programed conditions and use the results from carefully considered experiments to establish reasonable practical operating conditions for SFC. When this is done a reasonable picture emerges as to the usefulness of the most common LC columns and column packing types for packed-column SFC. The question is a significant one, as commercially available LC columns are used almost universally in the contemporary practice of SFC and the results obtained would be prejudicial to the growth of SFC if this choice proves inappropriate.

EXPERIMENTAL

All chemicals and solvents were of the highest purity available. Polyethylene glycol and polypropylene glycol standards were obtained from BASF (Wyandotte, MI, U.S.A.). Spherisorb alumina (20 μm and 100 μm) and Spherisorb ODS1 (5, 10, and 100 μm) octadecylsilanized silica were obtained from Phase Separations, (Queensferry, U.K.). Octadecylsilanized silica (40 μm) was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Corasil and PC18 pellicular silica and octadecylsilanized silica (all 37–55 μm) were obtained from Waters Assoc. (Framingham, MA, U.S.A.) and Alltech (Deerfield, IL, U.S.A), respectively. Carbopack B packings of various mesh sizes were obtained from Supelco (Bellefonte, PA, U.S.A.). Porapak Q polymeric beads (100–120 mesh), Seragen latex particles (6.4 $\pm$ 1.9 μm), Vydac wide-pore octadecylsilica (330 Å, 10 μm), Nucleosil silica (500 Å, 10 μm) and parafilm tape, were obtained from Anspec (Ann Arbor, MI, U.S.A.). Delta bond cyanopropyl- and octadecylsilica prepacked columns of 10 cm x 1 mm I.D. and 5 and 10 μm particle diameters were obtained from Keystone Scientific (State College, PA, U.S.A.). Ethoquad 18/25 was obtained from Armak Industrial Chemicals (Chicago, IL, U.S.A.).

All chromatographic experiments were performed using a Suprex 200A (Pitts-

burgh, PA, U.S.A.) supercritical-fluid chromatograph with either a tapered, fused-silica restrictor or a stainless-steel restrictor, prepared as described below. Chromatograms were recorded with either a Hewlett-Packard 3396A computing integrator (Avondale, PA, U.S.A.) or a Shimadzu R-111 chart recorder (Columbia, MD, U.S.A.). The mobile phase was supercritical-fluid-grade carbon dioxide from Scott Speciality Gases (Plumsteadville, PA, U.S.A.).

Since this project required installing and removing a large number of columns a rugged restrictor was required that incorporated a standard column end fitting. All results reported in this paper were obtained with the same restrictor, prepared from a 30 cm × 0.01 in. I.D. 1/16 in. O.D. stainless-steel capillary tubing. One end of the tube was symmetrically crimped with a pair of compound pliers. The flow-rate was adjusted by placing the tip of the restrictor in a small vial, filled with water, and adjusting the degree of crimping until the desired bubble rate was obtained. This corresponded to an atmospheric flow-rate of 8 ml/min of carbon dioxide at a pressure of 80 atm, set at the pump. A standard 1/16-in. fitting was then attached to the restrictor, which was positioned so that its tip was about 1 in. below the flame tip and the flame was not extinguished when the pump was operating at its maximum pressure. Excess capillary tubing was cut off. The detector base heater, which also heats the restrictor, was set to 300°C and remained at that temperature for all experiments.

Columns for SFC were prepared by slurry packing under high pressure, as used for LC and described in detail elsewhere[19], by the tap-and-fill method, similar to columns prepared for GC[20], and by displacement with supercritical carbon dioxide as described below. Standard Valco fittings, 2-μm screens, and precut, smoot-bore, stainless-steel tubing were used for all columns (Valco Instruments, Houston, TX, U.S.A.).

For the displacement packing method, the analytical column was connected directly to an appropriately sized reservoir, using a Valco union of larger bore than the internal diameter of the analytical column. Initially the reservoir was filled with excess bulk packing material by tap-and-fill using an electronic engraving tool for vibration; a water aspirator vacuum was applied to the base of the reservoir if necessary. The reservoir and the column were then connected and this assembly was connected to the supercritical-fluid chromatograph at its inlet as if it were a column (Fig. 1). At this point the packing assembly was still isolated from the supercritical fluid. The syringe pump was charged to 480 atm, and the pump isolation valve was opened, thereby flooding the packing assembly with high-velocity carbon dioxide. For a time of several minutes after the isolation valve was opened, the packing assembly was vibrated with the engraving tool. The initial flow-rate of carbon dioxide is relatively slow (the pump can almost recover the preset pressure setting), but as the bed begins to consolidate (usually < 1 min) the flow-rate of carbon dioxide will begin to increase and finally reach a constant value. After several milliliters of carbon dioxide (as read at the pump, high pressure) had passed through the column, a plug was placed in the outlet of the column so that the flow was restricted. The flow was maintained slow enough so that the pump could recover to the set point, 480 atm. This condition was maintained for a short period, and then the plug was removed and the syringe pump isolation valve was closed. After the packing assembly had depressurized through the column exit, the packing assembly was removed from the chromatograph and disassembled. Surplus packing at the top of the column was removed with a razor blade,

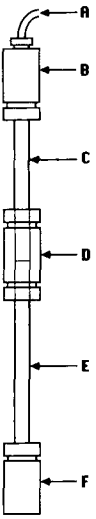


Fig. 1. Assembly for packing columns by the displacement method. A = high pressure carbon dioxide from SFC pump; B = Valco 1/8 in. to 1/16 in. reducing union; C = packing reservoir, 2.1 mm I.D., 1/8 in. O.D. stainless-steel tube; D = Valco bored through 1.8 in. union; E = column; and F = Valco column end fitting 1/8 in. to 1/16 in. fitted with a 2-μm screen.

and a screen and an end fitting were attached to the column. We have used the above method to pack columns with spherical particles from 5 to 40 μm in diameter, lengths from 3 to 30 cm, and column internal diameters from 0.5 to 5.0 mm. We have had columns in continuous service for several months without observable loss in efficiency.

Packings deactivated with Ethoquad 18/25 were prepared with the aid of a rotary evaporator. A dichloromethane solution of Ethoquad 18/25 was added to the required weight of packing material, and the solvent was very carefully evaporated. For particles less than 10 μm in diameter, some loss of material in the solvent vapors is unavoidable, and a different coating procedure may be more appropriate.

RESULTS AND DISCUSSION

In studies of column design for GC or LC, it is conventional practice to construct Van Deemter curves, curves employing reduced parameters according to Knox, or to make measurements of plate heights etc., and to relate the magnitude of these parameters to column properties of realistic column models. This approach is not successful with SFC as the relationship between mobile-phase density, viscosity, velocity, etc. are very complex for packed columns and are still poorly understood, even for the simplest case of operation at constant inlet density[9–14]. Since operation at constant density is not very rewarding in terms of the quality of separations obtained, pressure or density programming is virtually always used in practice, even for relatively simple mixtures. However, under these conditions, we know even less concerning the relationships between mobile phase variables and their changes with position along the column. Solution precipitation/dissolution and peak-compression phenom-

ena also have to be accounted for in a way that is unique. Thus, in this paper we will adopt an empirical approach to establish the influence of column length, column internal diameter, particle size, pore size, packing density, phase ratio, and surface activity on the properties of packed columns under pressure programed conditions.

Fig. 2 shows three separations of the dichloromethane-extractable portion of parafilm (a mixture of hydrocarbons from *ca.* C_{19} to C_{36}) on three columns, packed with pellicular, octadecylsilanized packing of different lengths. Increasing the column length from 4 to 15 cm produces a dramatic increase in resolution but increasing the column length from 15 to 30 cm results in only a small further increase in resolution, gained at the expense of increased analysis time. With decreasing particle size a similar effect is observed, and very long columns seem to offer few advantages in SFC. Columns in the range 10–25 cm are a reasonable compromise. Resolution for a fixed particle size is independent of the column diameter, at least in the range investigated, 0.5–5.0 mm. At larger internal diameters, the high linear velocity and mass flow-rate of the decompressed carbon dioxide at the detector causes problems in maintaining a steady flame. Columns of 1.0–2.0 mm I.D. are convenient to work with and will accept sample sizes in the microliter range without loss of column performance.

Fig. 3 shows the influence of particle size at a fixed column length on the chromatograms of the parafilm sample. The coarse 100-μm diameter particles produce very poor separations, while the difference between the slurry-packed 5-μm particle column and the tap-and-fill pellicular column, containing 37–55 μm particles

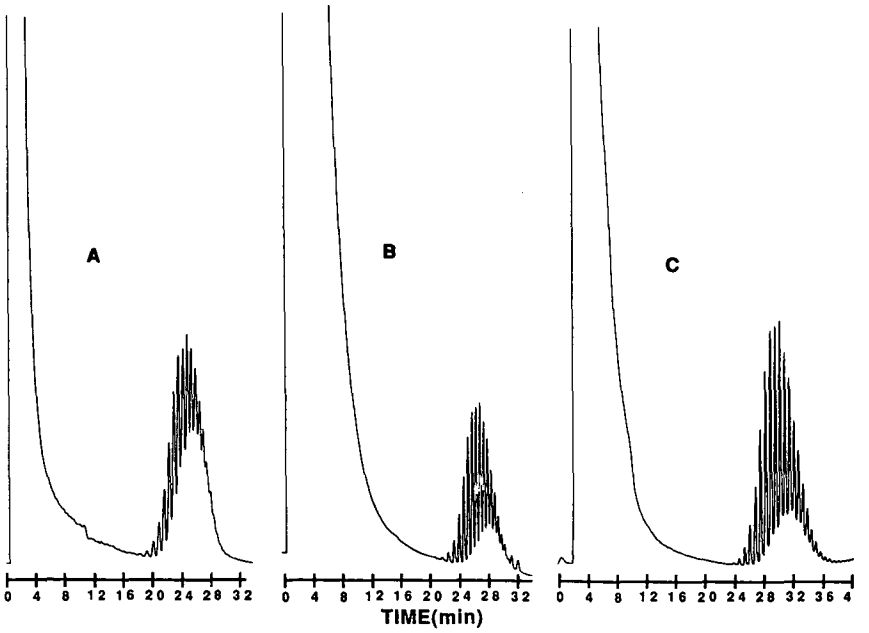


Fig. 2. The influence of column length on the resolution of a mixture of hydrocarbons extracted from parafilm with dichloromethane. The column lengths were A = 4 cm, B = 15 cm, and C = 30 cm, all of 1 mm I.D. Each column was packed with Alltech PC18 (37–55 μm) by the tap-and-fill method. The samples were separated at 80°C, using carbon dioxide as the mobile phase and the following pressure program: 80 atm for 10 min and then linearly increased to 400 atm over 30 min.

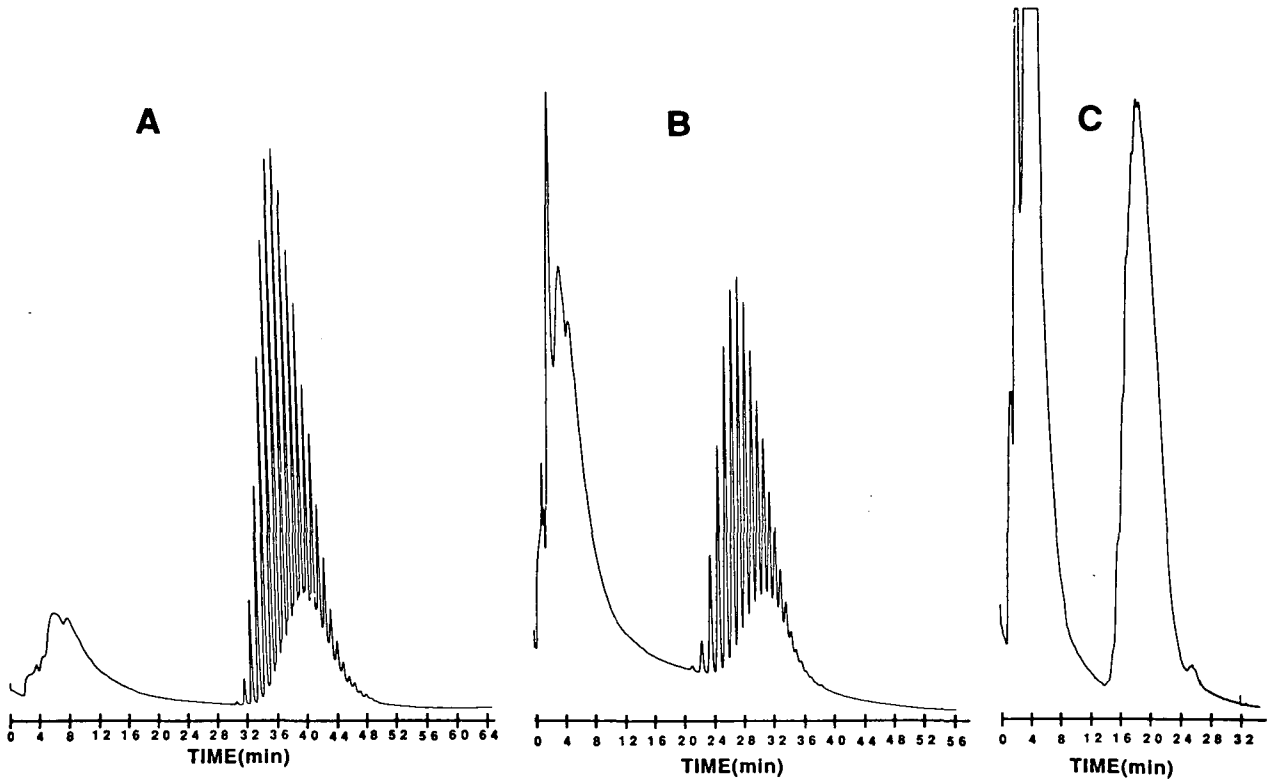


Fig. 3. The influence of particle size on the resolution of a mixture of hydrocarbons, extracted with dichloromethane from parafilm. Each column was 10 cm × 1.0 mm I.D. and carbon dioxide was used as the mobile phase with the following pressure program: 80 atm for 10 min and then increased linearly to 400 atm over 30 min at 80°C. Column A was obtained from Keystone and was packed with 5 μm particles using a liquid slurry technique; column B contained pellicular packing (37–50 μm) and column C 100 μm particles. Columns B and C were packed by the tap-and-fill method.

is not so great. Very large particle diameters are not very useful, even though they provide the lowest column pressure drop, while the smallest particles are not necessarily a great improvement over particles of intermediate size, because they increase the column pressure drop. Particle diameters in the range $5 < d_p < 10$ offer the highest separation capability, but with particle diameters up to 50 μm the differences are not as large as those commonly observed in LC.

To test the possibility that better use of the column pressure drop might be oberved by using a short, narrow, small-particle-diameter column connected to a coarser-particle-diameter column to provide a longer coupled column, two columns containing 10- and 40-μm diameter packings were connected in series in both combinations, Fig. 4. As can be seen the chromatograms are virtually identical. The separation is poor because the particle-size distribution of the 40-μm material is larger than is desirable. The entire column is clearly involved in the separation process, and segregated columns with a short, high-efficiency section, backed by a section of low

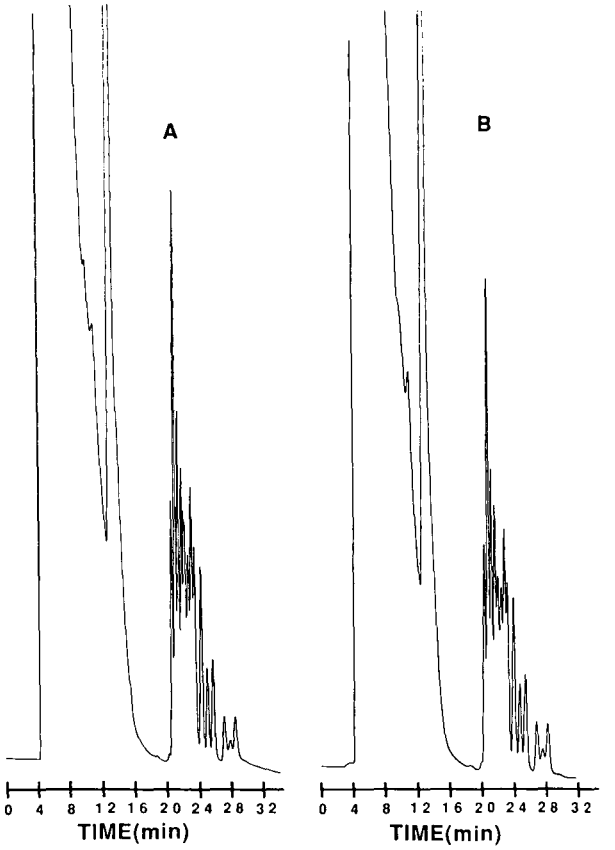


Fig. 4. Coupled columns: A = 10 cm (10 μm) + 10 cm (40 μm) and B = 10 cm (40 μm) + 10 cm (10 μm). Both columns (1.0 mm I.D.) were packed by tap-and-fill with Spherisorb ODS1 (10 μm) and Baker ODS silica (40 μm). The sample was a synthetic mixture of C_{19} to C_{40} *n*-alkanes. The temperature was 100°C. The mobile phase was carbon dioxide with the following pressure program: 80 atm for 10 min then increased linearly to 400 atm over 30 min.

pressure drop, in which the resolution of the sample is enhanced by its migration through a zone of low or no interaction with the stationary phase are not a tenable or useful model. Columns should be homogeneously packed with a single particle size for optimal results, and, as in GC, and LC, the performance of series coupled column most closely resembles that of the lowest performance obtained for the individual columns.

Many separations in SFC have been performed to separate oligomeric mixtures. Fig. 5 shows the separation of a series of polyethylene glycols with an average molecular weight of 400, 600, and 1000 on a pellicular octadecylsilanized packing, deactivated with Ethoquad 18/25. The low-molecular-weight standard is resolved normally, of the next higher-molecular-weight standard only the low-molecular-weight fraction is resolved, and the high-molecular-weight standard is unresolved. The hydrodynamic radius of the oligomers is much smaller than the pore size of the packing (assuming that the same meander spiral model for the oligomers in solution is applicable to their structure in a supercritical fluid) and it can reasonably be assumed that the oligomers of higher-molecular-weight are not excluded by size[21,22]. Fig. 6 shows the separation of polyethylene glycols of average molecular weight 600 and 1000 on the wide-pore (330 Å) octadecylsilanized silica packing deactivated with Ethoquad 18/25. The 600-molecular-weight standard is now normally resolved, while the 1000-molecular-weight standard shows normal resolution of the early fraction

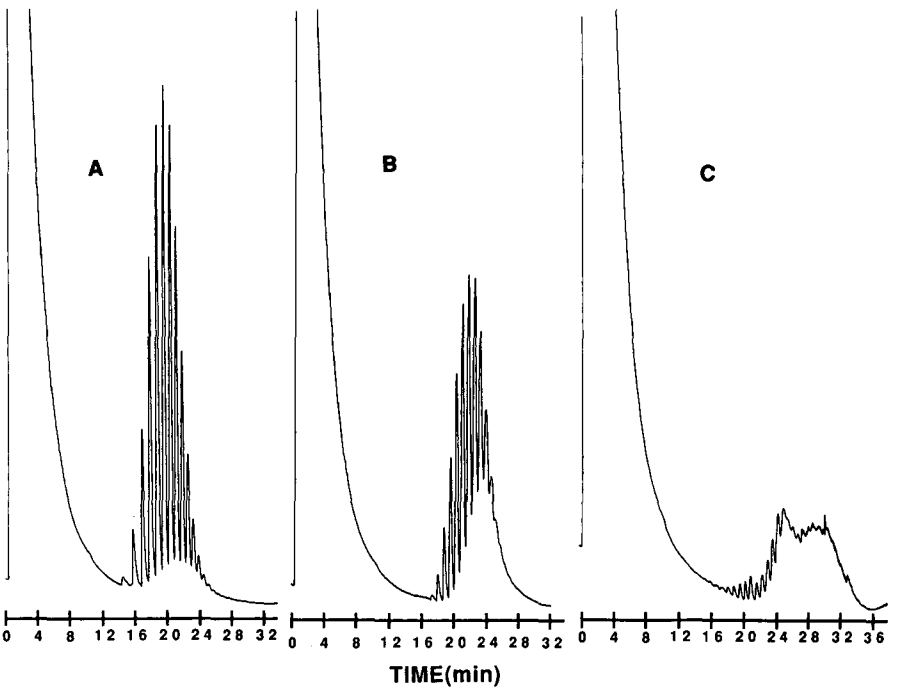


Fig. 5. The separation of polyethylene glycols of average molecular weight, A = 400, B = 600, and C = 1000. The column was 15 cm × 1 mm I.D. packed by tap-and-fill with Alltech PC18 + 0.4% (w/w) Ethoquad 18/25. The mobile phase was carbon dioxide; temperature 80°C, and the pressure program, 10 min at 100 atm, increased linearly to 400 atm over 30 min.

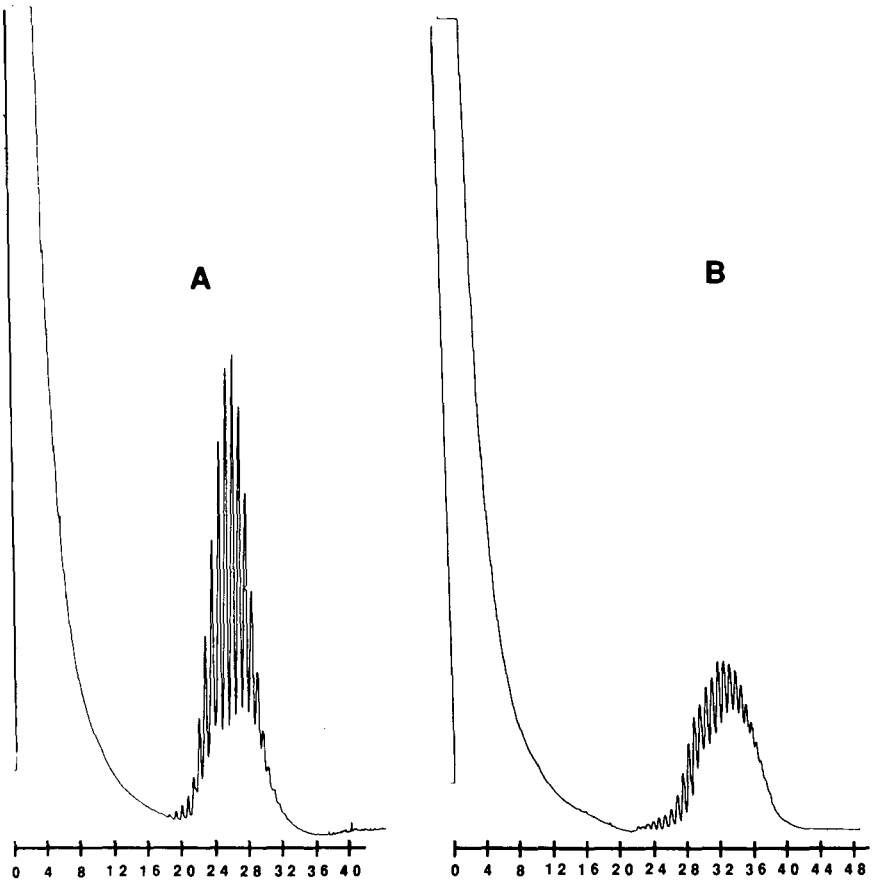


Fig. 6. Separation of standards of polyethylene glycol with an average molecular weight A = 600 and B = 1000 on a wide-pore octadecylsilanized packing (Vydac, 330 Å) coated with 3.5% (w/w) Ethoquad 18/25 and particle size 10 μm. The column, 10 cm × 1 mm I.D. was packed by the displacement method. The mobile phase was carbon dioxide, temperature 80°C, and the pressure program 10 min at 100 atm increased linearly to 400 atm over 30 min.

and poor resolution of the high-molecular-weight fraction. Since the elution density range and temperature are the same the improved separation capacity must result from an increase in efficiency, but more importantly, an increase in the selectivity of the chromatographic system between the two types of column packings. Fig. 7 shows the separation of a polypropylene glycol standard with an average molecular weight of 3010, contaminated with a small amount of polypropylene glycol with an average molecular weight of 1000. In this case, the low-molecular-weight contaminated is well resolved, but the high-molecular-weight major component is unresolved. More selective chromatographic conditions are needed to distinguish between oligomers in the high-molecular-weight range, although the polymers themselves are easily eluted (unseparated) to well over 4000 in molecular weight. For perspective, polypropylene glycol of molecular weight 3010, has an oligomer number of 51, and thus, the oligomer range with an average number of 51 repeat units represents very small changes in

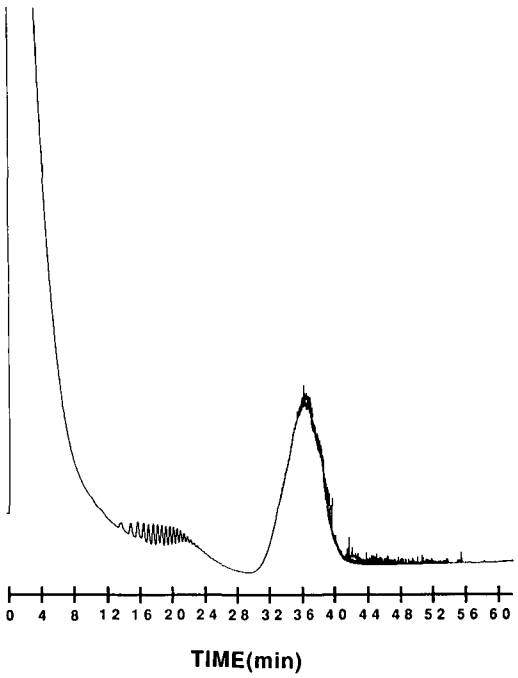


Fig. 7. Separation of a polypropylene glycol standard of average molecular weight 3010, contaminated with some oligomers of lower-molecular-weight material. The column was 7 cm × 1 mm I.D. The other conditions are identical with those for Fig. 6.

molecular characteristics. It will be very difficult for oligomer separations to match the elution capability and separation capacity of the column for high-molecular-weight polymers with large oligomer numbers.

To make the most practical use of the available elution density the density drop along the column must be minimized. To maintain reasonable efficiency small-particle packings are required and this leaves the packing density as the only variable that can be changed. The tap-and-fill method provides good columns of high permeability for particles down to about 20 μm and, with increasing experimental difficulty in packing, down to about 10 μm. The displacement method with the use of supercritical-fluid carbon dioxide is convenient for preparing columns of moderate packing density in the 5–20 μm particle-size range that are difficult to pack consistently by the tap-and-fill method. Fig. 8 shows a separation of hydrocarbon standards on a liquid-slurry-packed and a supercritical-fluid-packed column of 5 μm particles. The small differences in resolution between the two columns are due to the use of different packing materials. The supercritical-fluid-packed columns are more permeable and as efficient as the slurry-packed columns. The former column type is stable to supercritical-fluid conditions, indicating that columns need only be packed as densely as is dictated by the stress they will experience in operation. Supercritical-fluid-packed columns sediment too rapidly to be of use in LC or to permit any comparison of reduced-plate height- and flow-resistance measurements to be made. For SFC applications they provide more economic use of the available column inlet pressure, pro-

Fig. 8. Comparison of column types, packed by the displacement method (A) with supercritical carbon dioxide, and (B) by the liquid slurry technique. Column A was 10 cm × 1 mm I.D. packed with 5 μm Spherisorb ODS1, Column B was 10 cm × 1 mm I.D. packed with 5 μm Nucleosil ODS. The sample was a synthetic mixture of *n*-alkanes, C_{19}–C_{40}; mobile phase, carbon dioxide, temperature, 100°C, and pressure program, 10 min at 100 atm increased linearly to 400 atm over 40 min.

vide a lower pressure (density) drop per unit column length without loss of column performance, and should, at least in theory, permit the elution of higher-molecular-weight analytes than densely packed columns containing particles of the same size.

Column packings commonly used in LC have large surface areas which may not be required for separations in SFC. Large surface areas generate low phase ratios, and this leads to high retention. This may not be ideal for eluting middle-molecular-weight analytes. Secondly, packings with a large surface area contain a greater number of active sites per unit weight and will be more difficult to deactivate. SFC is being championed for the separation of polar and labile molecules that are likely to interact unfavorably with active column packings, resulting in peak asymmetry and possibly adsorption or catalytic transformation. Hirata[23,24] has reported that silica becomes irreversibly modified when ethanol-containing mobile phases are used in SFC and Schmitz *et al.*[25] have shown that silica reacts chemically with 1,4-dioxane under supercritical-fluid conditions. Doehl *et al.*[26] found that carboxylic acids, amines, and

amides are strongly adsorbed on commercially available column packings in SFC. Most of these reactions are associated with the silanol groups which are known to react chemically with alcohols, amines, and isocyanates at moderate temperatures to form bonded ligands[27]. An interesting example of chemical reactions occurring on silica is shown in Fig. 9 for the separation of cholestane, 5-cholestene, 3,5-cholestadiene and cholesterol at two different temperatures on a low-surface-area pellicular silica packing. Adsorption interactions are useful for the separation of the three cholestane hydrocarbons which are difficult to resolve on bonded-phase packings. On the other hand, the peak width for cholesterol is substantially broadened and shows tailing on its leading edge. Increasing the column temperature causes an increase in tailing and a loss of injected mass. Increasing the column temperature further can result in the complete abstraction of cholesterol from the chromatogram. The interaction of proton-donor solutes with residual silanol groups occurs in our experience on the most chemically deactivated bonded-phase supports commercially available, and is due to chemical interaction rather than adsorption, since the effect is more pronounced at elevated temperatures. More inert packing materials than are generally available at present will be needed for the separation of polar solutes in packed-column SFC. Packings of low surface area should be used, as they provide a lower column activity after deactivation.

Liquid organic salts have been used in GC for the analysis of polar com-

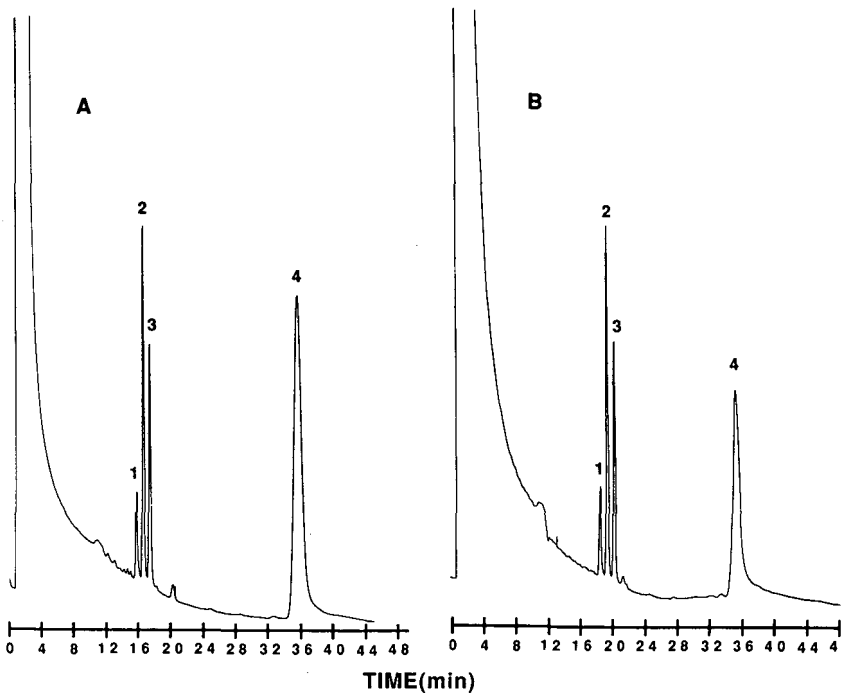
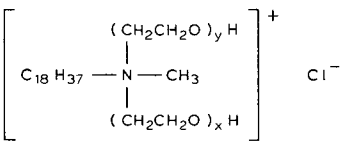


Fig. 9. Influence of silanol groups on the separation and recovery of 1 = cholestane; 2 = 5-cholestene; 3 = 3,5-cholestadiene, and 4 = cholesterol at A = 80°C and B = 100°C. The column was 10 cm × 1 mm I.D. packed with Corasil (II) by the tap-and-fill method. The mobile phase was carbon dioxide and the pressure program 10 min at 80 atm increased linearly to 300 atm over 30 min.

pounds[28–32]. Several salts with low melting points and liquid ranges greater than 100°C are known. These salts are excellent column deactivating agents for diatomaceous supports. For use in SFC, the liquid organic salts must be non-extractable at the highest density of the mobile phase to be used. If this condition could be met, liquid organic salts would provide a simple means of stationary-phase modification and support deactivation. One such useful salt is Ethoquad 18/25, stearylmethyldipolyoxyethylammonium chloride of average molecular weight 994 ($x + y = 15$), shown below.



Ethoquad 18/25 is a liquid at room temperature and has been used in GC for the separation of essential oils and other polar compounds at temperatures up to 300°C, where it shows a chromatographic selectivity similar to Carbowax 20M[33]. Fig.

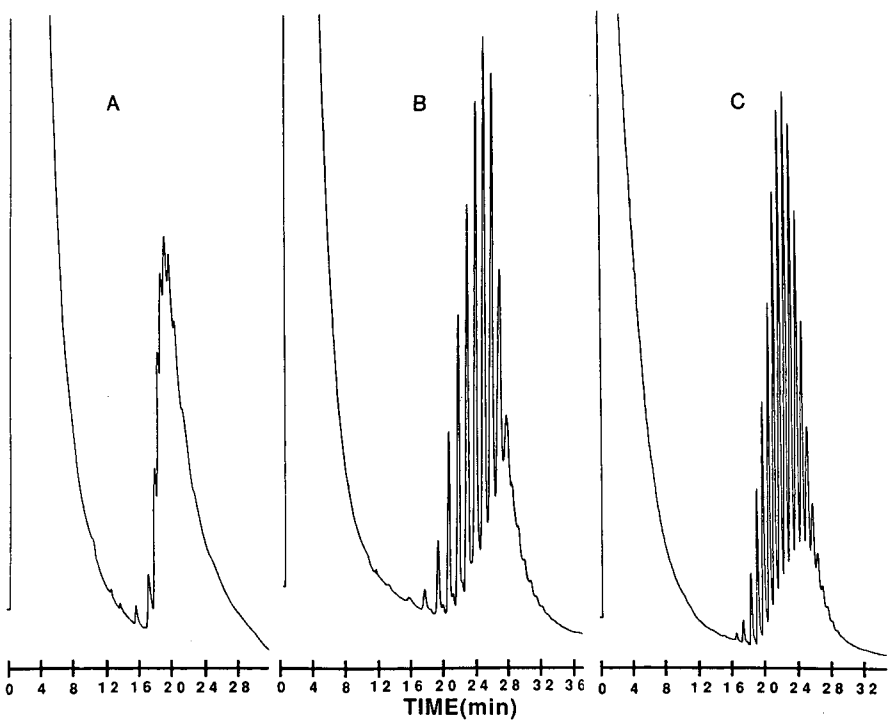


Fig. 10. Separation of Triton X-100 on a 15 cm × 1 mm I.D. column of (A) Alltech PC-18; (B) a 15 cm × 1 mm I.D. column of Alltech PC18 coated with 0.4% (w/w) Ethoquad 18/25; and (C) a 7 cm × 1 mm I.D. column of Vydac octadecylsilanized silica of 10-μm particle diameter and 330 Å nominal pore diameter coated with 3.6% (w/w) Ethoquad 18/25. Columns A and B were prepared by tap-and-fill, and column C by the displacement method. The mobile phase was carbon dioxide, temperature, 80°C, and pressure program, 10 min at 100 atm increased linearly to 400 atm over 30 min.

10 shows a comparison of the separation of Triton X-100 on a pellicular octadecylsilanized packing with and without coating with 0.4% (w/w) Ethoquad 18/25 and on a wide-pore octadecylsilanized silica packing coated with 3.6% (w/w) Ethoquad 18/25. Without Ethoquad, Triton X-100 (which has an approximate average molecular weight of 600) is unresolved on the uncoated pellicular packing but well resolved, as far as the early fraction of the oligomers is concerned, on the Ethoquad 18/25-coated packing. On the wide-pore material, better resolution of the higher-molecular-weight oligomers is achieved, in part due to the use of smaller-diameter particles. In both cases, the separation benefits from the combined deactivation and selectivity modification of the stationary phase, brought about by the use of the liquid organic salt.

Liquid organic salts at high phase loadings are useful stationary phases, which can provide true partitioning interactions. Fig. 11 shows the separation of Triton X-100 on a silica-based packing, heavily loaded with Ethoquad 18/25. Compared to Fig. 10, the resolution is improved, particularly for the heavy oligomers. Fig. 12 shows a separation of cholestane, caffeine, and cholesterol on the same column. Caffeine and cholesterol are difficult to elute at these low concentrations from bond-

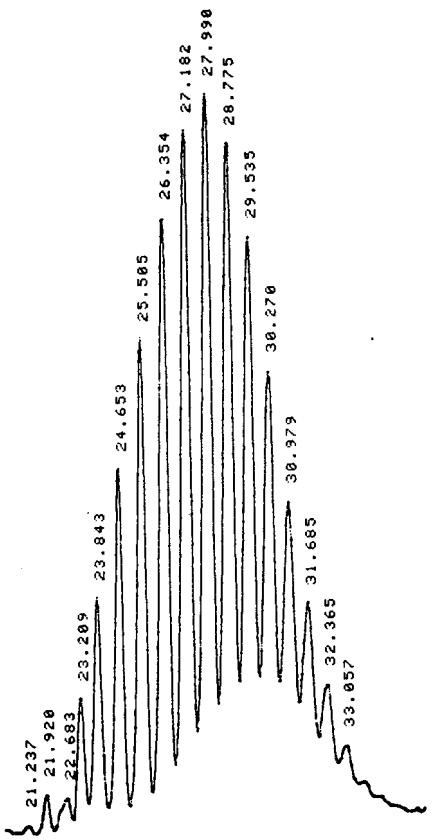


Fig. 11. Separation of Triton X-100 on a 10 cm × 1.0 mm I.D. column of Nucleosil 500, 10 μm particle size, coated with a heavy loading of Ethoquad 18/25 (*ca* 20% w/w). The mobile phase was carbon dioxide, temperature, 80°C, and pressure program, 10 min at 80 atm, increased linearly to 450 atm over 30 min.

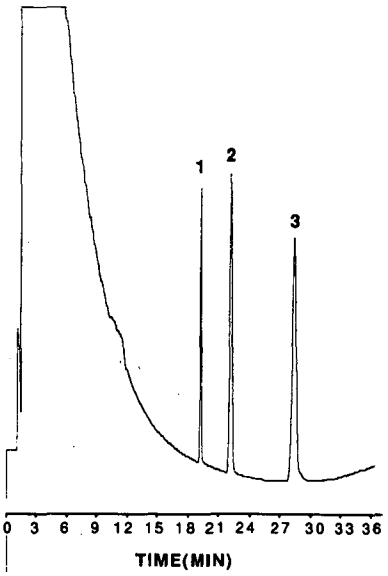


Fig. 12. Separation of 1 = cholestane, 2 = caffeine, and 3 = cholesterol on the same column and the same pressure program as that used for Fig. 11.

ed-phase columns without significant tailing. The heavily loaded Ethoquad 18/25 column shows excellent efficiency and good peak shape. The liquid organic salts may soon find a significant role in the development of partition-based columns of variable selectivity for packed-column SFC.

The problems associated with the chemical and adsorptive activity of silica- and alumina-based packings under supercritical-fluid conditions could be circumvented by using materials with a different structure. Graphitized forms of carbon and polystyrene divinylbenzene macroporous polymers were considered likely candidates. Columns prepared from Carbopack B showed poor mechanical stability, lower-than-expected efficiencies, and short column lifetimes. Stronger forms of porous graphitic carbon, such as those described by Knox *et al.*[34] might be more suitable. Porous polymers, based on polystyrene divinylbenzene, (Porapak Q, PRP-3, and Seragen) were also found to be physically unstable to density changes of the mobile phase. Usually, if the column is opened after pressure programming, the packing will exude from the column like toothpaste from a squeezed tube. As it reaches equilibrium, it will then shrink back into the column. The swelling of cross-linked polymers in supercritical fluids is not unprecedented, as this behavior is noted for cross-linked siloxane polymers used as stationary phases in open-tubular-column SFC[35,36]. There may be a threshold density below which a particular macroporous polymer may not swell and could be used for SFC. However, this would certainly significantly reduce the molecular weight range of analytes that could be separated. The Hamilton PRP-3 material has good LC properties and is at least stable to aqueous organic solvents[37]. This points to the fact that the properties of packings for SFC are not necessarily identical to those observed in LC.

CONCLUSIONS

It is clear from the above discussion that high-performance column packings developed for analytical LC are not ideal for SFC. Prepacked LC columns that have been used in SFC typically have lengths of 10–30 cm, internal diameters of 1.0–5.0 mm, particle sizes of 5–10 μm, pore sizes of 60–150 Å, and surface areas from 100–500 m^2/g. The available column lengths seem reasonable for SFC and the smaller internal diameters of 1.0–2.0 mm are a reasonable compromise between sample loadability and experimental difficulties related to the convenience of instrument design and flame stability (when the flame ionization detector is used). Particle sizes of between 5 and 10 μm are also reasonable in terms of efficiency, although we suspect that the optimum particle size may be closer to 10 than 5 μm. Here, there is a very complex relationship between the column pressure drop, the mobile phase velocity profile along the column, the length of the column, the analyte diffusion coefficients, column temperature, and particle size. To make the most economic use of the available column inlet pressure (density) and column length, the column permeability should be no lower than is dictated by the need to maintain the average column efficiency. This dictates that columns should be less densely packed than is current practice for LC columns. Fortunately, this can be achieved by using the supercritical fluid itself as the packing solvent. This method is simple, has a very low column failure rate, and is by no means as difficult to master as procedures for packing LC columns. There should be little deterrent to adopting the described packing procedure in those laboratories that do not pack their own LC columns.

Greater consideration should be given to the use of wide-pore packings in SFC as a means of lowering the surface area of macroporous packings. No commercially available column packing we have investigated is sufficiently inert for the analysis of all types of polar molecules. Even the most highly silanized and chemically bonded silica supports still contain unreacted silanol groups in appreciable numbers. These groups can condense with proton donor solutes with the elimination of water (chemical reaction) and behave as proton donor solutes to analytes with proton acceptor groups, *e.g.* amines. The difficulty of deactivating diatomaceous supports, which typically have surface areas of 0.5–4.0 m^2/g, to reduce tailing and chemical interactions in GC is well known. Even so-called wide-pore packings used in LC with pore diameters of 500–1000 Å will typically have surface areas between 25–35 m^2/g –already an order of magnitude greater than typical supports used in GC. The adequate deactivation of these column packings will be difficult, but it will only be more difficult for column packings of higher surface area. One approach we have introduced in this paper is the use of liquid organic salts as support masking agents and stationary phase modifiers to adjust selectivity. Another avenue is to seek out less active materials than silica- or alumina-based packings that can withstand the mechanical and physical properties of supercritical fluids. Commercially available silica- and alumina-based packings in current use may not only mar the aesthetic aspects of chromatograms of polar molecules in high concentration, but they may also preclude attempts at trace analyses for the same compounds due to a low recovery of injected material. Derivatization has been very successful in GC in solving problems of this kind and should be seriously considered when developing separations by SFC. At the present state of column technology it may be far less challenging to change the char-

acter of the analyte than the column packing. There may be additional benefits in this approach to enhancing the solubility of polar analytes in supercritical-fluid carbon dioxide.

We have found two kinds of column packings most useful in our studies. Pellicular packings are relatively inexpensive, easily packed into columns by tap-and-fill methods, and have low surface areas and moderate activity. They are convenient for screening unknown samples, because they can be quickly prepared and the packing can be discarded if changed in character by the sample at only a very small fraction of the cost of small-particle columns. Their efficiency is not so low as might be anticipated from a LC point of view. For optimum chromatographic performance, small particle, (10 μm or thereabouts) spherical particles, packed by the supercritical-fluid displacement method and having a wide-pore/low surface area are preferred.

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CHROMSYMP. 1558

ANALYSIS OF VOLATILES IN TALL OIL BY GAS CHROMATOGRAPHY, FLAME-PHOTOMETRIC DETECTION, FLAME-IONIZATION DETECTION AND MASS SPECTROMETRY

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SUMMARY

Tall oil is a by-product obtained from the southern kraft pulping process. In order to isolate and detect the odor compounds in tall oil, we concentrated the volatiles, using a gas-phase stripping and adsorption on a thermally stable porous polymer. The volatiles were thermally desorbed onto a high-resolution capillary column. The separated compounds were detected simultaneously by flame-ionization detection and specific sulfur detection. Capillary gas chromatography–high-resolution mass spectrometry was used for positive confirmation of the eluted compounds.

More than a hundred compounds were identified, about twenty of which were sulfur-containing compounds, causing the malodor of the tall oil. The total sulfur content in tall oil was 470 ppm. Purging with inert gas at 250°C for 24 h removed only 60 ppm of the sulfur present. However, most of the bad odor was removed during the first 30 min of purging. This treatment also improved the color quality of the tall oil.

INTRODUCTION

Tall oil is the principal by-product of the sulfate pulping process of pine wood. Rosin acids and fatty acids represent more than 90% of tall oil, the rest being alcohols, esters, hydrocarbons and a complex mixture of other organic compounds at trace levels. This complex mixture is found in different locations in living trees[1,2]. In the pulping process, chipped wood is heated under pressure in the presence of sodium hydroxide and sodium sulfide. Cellulose and lignin are liberated, while fatty acids and rosin acids are changed to water-soluble soaps. This solution is known as "black liquor". This liquor is concentrated under reduced pressure and then treated with sulfuric acid, converting it to crude tall oil.

Most of the fatty acids present in tall oil are oleic and linoleic acids, while most of the rosin acids are present as abietic-type acids plus dehydroabietic acids. The ratio of fatty acids to rosin acids depends on the species and on the geographical location of the pine trees. For example, the rosin acid content of crude tall oil from the southeastern

U.S.A. is between 40% and 50%, while further north in Virginia, it is between 30% and 35% of the total tall oil[3].

About one million tons of crude tall oil are produced in the U.S.A. per year. The two principal distillable products of tall oil, tall oil fatty acids and tall oil rosin, have several applications, such as intermediates for manufacturing adhesives, inks, polyamide resins and epoxy resins. They also find use in protective coatings, soaps, flotation agents, and paper sizing. About 10% of the crude tall oil is used without fractional distillation for some specific applications, such as ore flotation and in oil well drilling muds[4].

The quality of tall oil is determined basically by the concentration of the acids, the unsaponified fraction and by the odor and color qualities. The odor quality of tall oil, as well as the odor of the gaseous emissions from pulp mills, are significant and complex problems. The source of the odor produced during manufacturing and in the final product is believed to be sulfur-containing compounds.

Treating black liquor with H_2SO_4 to liberate tall oil releases significant amounts of H_2S in the mill (*ca.* 0.27 lbs./ton of pulp)[5]. Some sulfur-containing compounds are formed during the pulping process. In the presence of sodium sulfide and sodium hydroxide, sulfide and hydrosulfide ions are formed. These ions react with methoxy groups of lignin to form methyl mercaptan[6] and dimethyl sulfide[4]. In the presence of O_2, methyl mercaptan can form dimethyl disulfide[4]. Other sulfur-containing compounds are probably formed, but these have not been investigated.

The types of organic sulfur-containing compound formed during Kraft pulping depend on temperature, the amount of sodium sulfide added, the pH, the design of the plant and the treatment time[5]. At lower temperatures, the amounts of methyl mercaptan formed exceed the amounts of dimethyl sulfide. An increase in temperature or in the amount of added sodium sulfide will cause an increase in the amount of organo-sulfur compounds liberated during the process[5]. Hydrogen sulfide and methyl mercaptan, being gases, are mostly removed during the pulping process. Therefore, they cannot be the major source of odor in tall oil. Other odor-causing compounds, whether they contain sulfur or not, have not been previously identified.

In this study, we developed a method for the concentration of volatiles in tall oil. Specifically, the volatiles were concentrated by gas-phase stripping, separated on a high-resolution capillary column, and detected simultaneously by flame-ionization detection (FID) and sulfur flame-photometric detection (FPD). Peaks were identified by high-resolution gas chromatography–mass spectrometry (GC–MS). We monitored the profile of volatiles released from tall oil over a period of 24 h.

EXPERIMENTAL

The apparatus used in this work separated the volatile components by gas-phase stripping[7]. It consisted of a glass tube (15 × 2 cm O.D.). The bottom part of this tube was attached to 6-mm-O.D. glass tubing to allow pure helium to bubble through the tall oil. A 15-cm condenser was adapted to this glass device, and chilled water was used to cool the condenser. A sample tube (110 × 10 mm O.D.) was placed on top of the condenser via shrinkable PTFE tubing. The sample tube was filled with *ca.* 150 mg of Tenax GC, 80–100 mesh (Alltech Assoc., State College, PA, U.S.A.). Two small plugs of glass wool kept the adsorbent in place. Before use, the adsorbent (Tenax GC) was

conditioned for several hours in a specially built all-metal device at a temperature of 350°C in a stream of helium[8]. All glassware was silanized by standard procedures and kept in an oven at 200°C.

A nominal sample size of 10 ml of tall oil was transferred to the glass device, which was then placed in an oil bath at a controlled temperature. All samples analyzed in this work were supplied by Arizona Chemical Co. (Tuxedo, NY, U.S.A.). The heated tall oil was purged with pure helium at a rate of 30 ml/min. The purge gas continuusly transferred the volatiles from the tail oil to the adsorbent. After collection, the trap was removed and placed in a thermal desorption system[8].

A small section of fused-silica capillary tubing (750 × 0.32 mm O.D.) was attached to the desorber and kept at liquid nitrogen temperatures. Through this pre-column of fused silica, coated with DB-5 (J&W Scientific, Folsom, CA, U.S.A.), helium was passed at 20 ml/min for 15 min. After desorption was completed, the pre-column was removed and placed in the gas chromatograph, where it was attached to the front end of the capillary column. The analytical column was fused silica (50 m × 0.34 mm O.D.), coated with immobilized SE-30 (J&W Scientific). The column was operated at linear gas velocity of 32 cm/s. The gas chromatograph was a Varian 3700, equipped for FID and sulfur FPD. The effluent of the column was split equally between the two detectors. The chromatographic parameters were as follows: detector temperature, 300°C; temperature program, 2 min at 40°C, then 5°C/min to 170°C.

The chromatographic signals were recorded and integrated on a Hewlett-Packard 3357 data system. The GC–MS studies were performed on a Kratos MS-50 high-resolution mass spectrometer equipped with a Perkin-Elmer Sigma 3 gas chromatograph. The mass spectrometer was operated in the high-resolution mode with a resolution of 6000 and at a scan rate of 1.5 s/dec. A Data General Nova 4 computer was used for the collection of data and the subsequent calculation of exact mass.

Total sulfur analysis was carried out by combustion in a Parr oxygen bomb, followed by measurement of sulfate ion by ion chromatography[9].

RESULTS AND DISCUSSION

Gas-phase stripping is a commonly used technique for the concentration of trace compounds in liquid samples. Subsequent analysis of the enriched material by high-resolution GC and GC–MS often provides information which cannot be obtained otherwise. Typical applications of these techniques include the analysis of volatile pollutants in water and the analysis of aromatic compounds in beverages.

We investigated the effects of the stripping temperature between 20°C and 250°C, and found that the efficiency of volatile stripping was poor at low temperature. At 20°C, the chromatogram of the collected sample did not change substantially after 8 h of purging with helium. At 250°C, stripping of volatiles was greatly enhanced. Fig. 1 shows the volatiles chromatogram recorded with FID and FPD after the volatile fraction was collected at 20°C for 30 min, and Fig. 2 shows the chromatogram after the volatile fraction was collected at 250°C for 2 min. Fig. 3 shows the GC–MS analysis of a sample collected at 250°C for 20 min, and Fig. 4 shows a typical expanded portion for the first 10 min of a GC–MS analysis.

Figs. 2–4 are very complex. Table I lists 120 compounds which were identified by

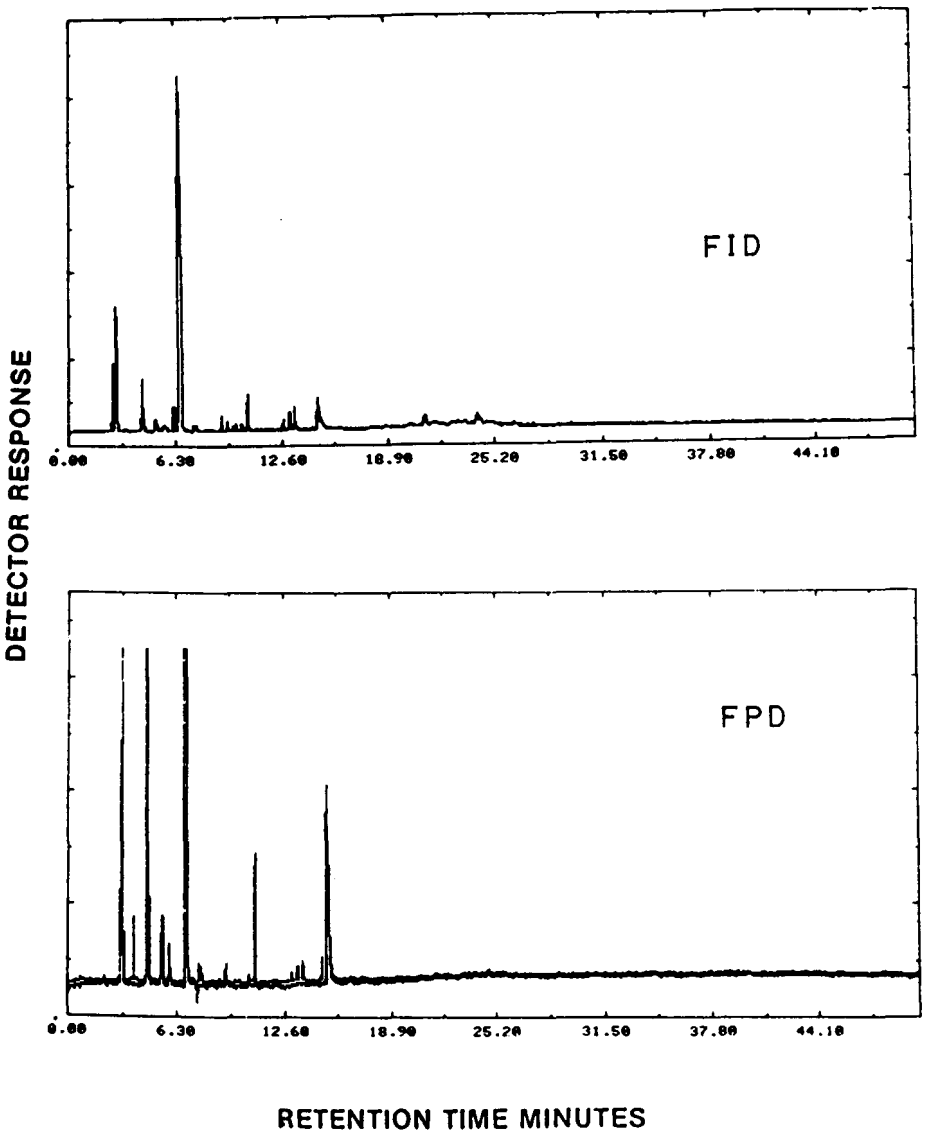

Fig. 1. Profile of tall oil volatiles, collected at 20°C for 30 min. The upper chromatogram was recorded by the flame-ionization detector and the lower chromatogram by the flame-photometric detector. For chromatographic conditions, see text.

MS from a typical volatile tall oil sample. These compounds include various hydrocarbons, sulfur-containing compounds, and oxygen-containing compounds. Identification of many of these was based on the exact measurement of molecular ions. High-resolution MS with a mass resolution of over 6000 and with mass accuracy of milli-mass units was essential for the identification of this complex mixture. In

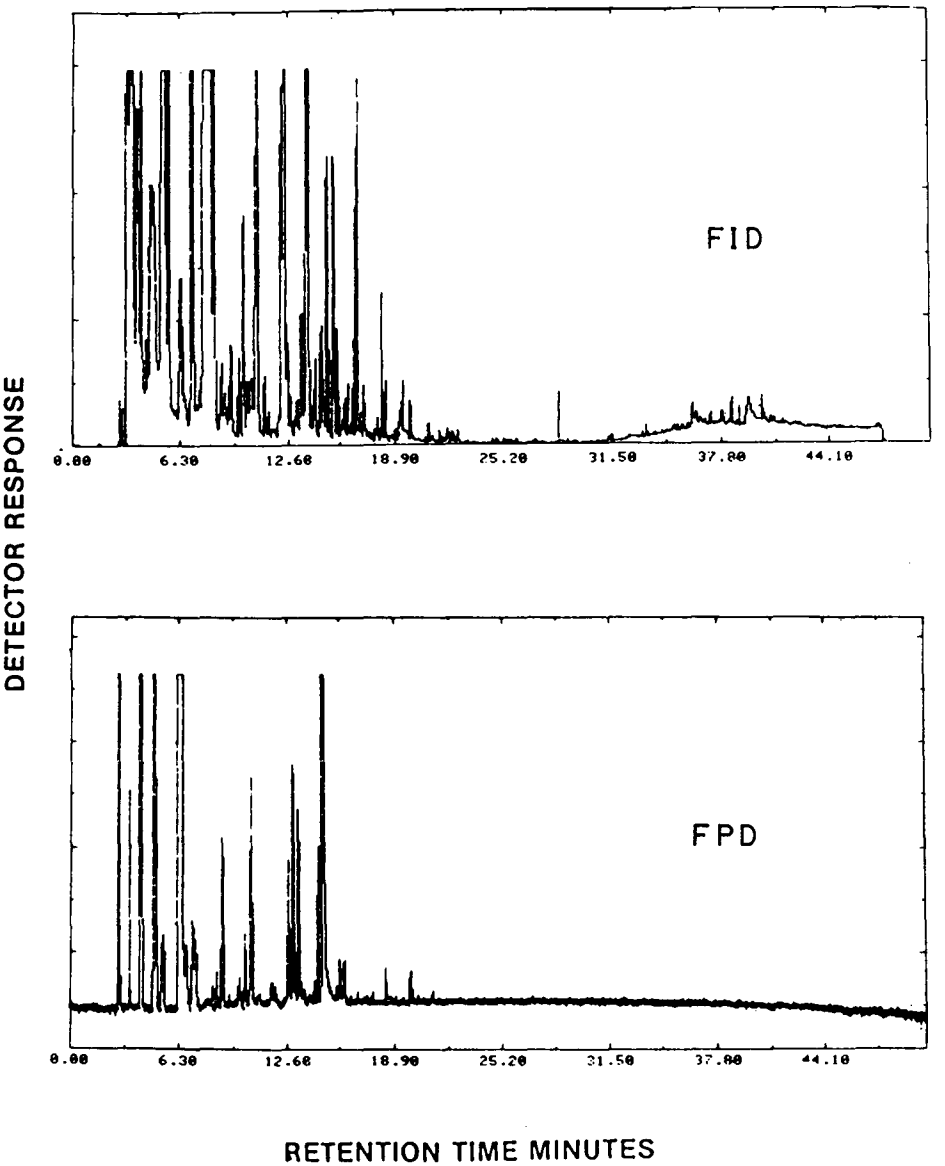

Fig. 2. Profile of tall oil volatiles, collected at 250°C for 2 min. For chromatographic conditions, see text.

addition to exact mass, the presence of ^{34}S isotope ions was also used for confirmation of sulfur-containing compounds.

Major peaks in Figs. 3 and 4 are numbered according to their elution order and are listed in Table I. Peaks labeled with plain numbers are hydrocarbons. Peaks labeled with bold (underlined) numbers are sulfur-containing compounds. The majority of identified compounds are hydrocarbons, mostly with a backbone structure of *ca.* C_{10} or C_{20} (terpenes are C_{10} compounds, and rosin acids are C_{20} compounds).

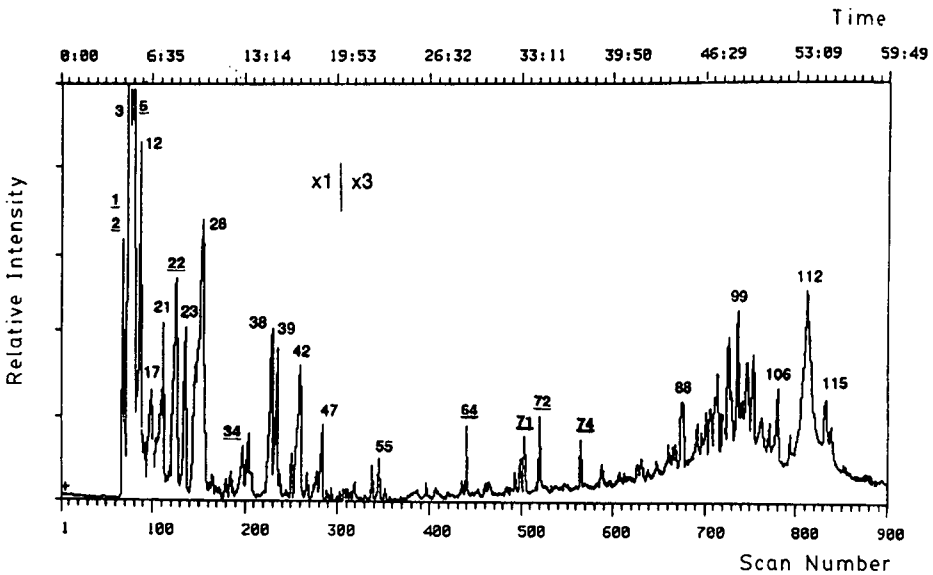

Fig. 3. GC–MS of tall oil volatiles collected at 250°C for 20 min. For chromatographic conditions, see text. Peaks labeled with plain numbers are hydrocarbons. Peaks labeled with underlined numbers are sulfur-containing compounds. Peak identifications are listed in Table I. Time in min:s.

Identification of these hydrocarbons is beyond the scope of this investigation and was not attempted.

In addition to hydrogen sulfide, 22 sulfur-containing compounds were identified in the sample. The amount of hydrogen sulfide retained in tall oil is probably not significant, since most of it is released during the pulping process. About 4.5% of the volatile sulfur compounds released to the atmosphere during the pulping process is methyl mercaptan and *ca.* 0.1% is dimethyl disulfide. Both compounds were detected in tall oil[4].

Other sulfur-containing compounds present in trace amounts are saturated alkyl mercaptans with carbon chains up to C_{10}. The higher-molecular-weight mercaptans probably contribute only insignificantly to the malodor because of their relatively small concentration and low volatility. Several oxygenated compounds were detected at trace levels, but no nitrogen-containing compounds were detected. Since hydrocarbons and oxygenated compounds are generally less malodorous, it may be safe to conclude that the offending odor is caused by combinations of various mercaptans, particularly the lower-molecular-weight alkyl mercaptans.

Fig. 5 shows the profile of organic volatiles released (total peak area) from tall oil over a 24-h period. For sulfur-containing compounds determined by FID, the amount of volatiles released decreased to constant but small amounts after *ca.* 1 h of purging with helium at 250°C. On the other hand, the profile of total volatiles recorded by FID initially increased, followed by a slight decrease with time.

The rapid loss of some of the sulfur-containing compounds is due to the loss of volatile sulfur-containing compounds. The persistent evolution of low levels of sulfur is due to slow decomposition of higher-molecular-weight sulfur-containing com-

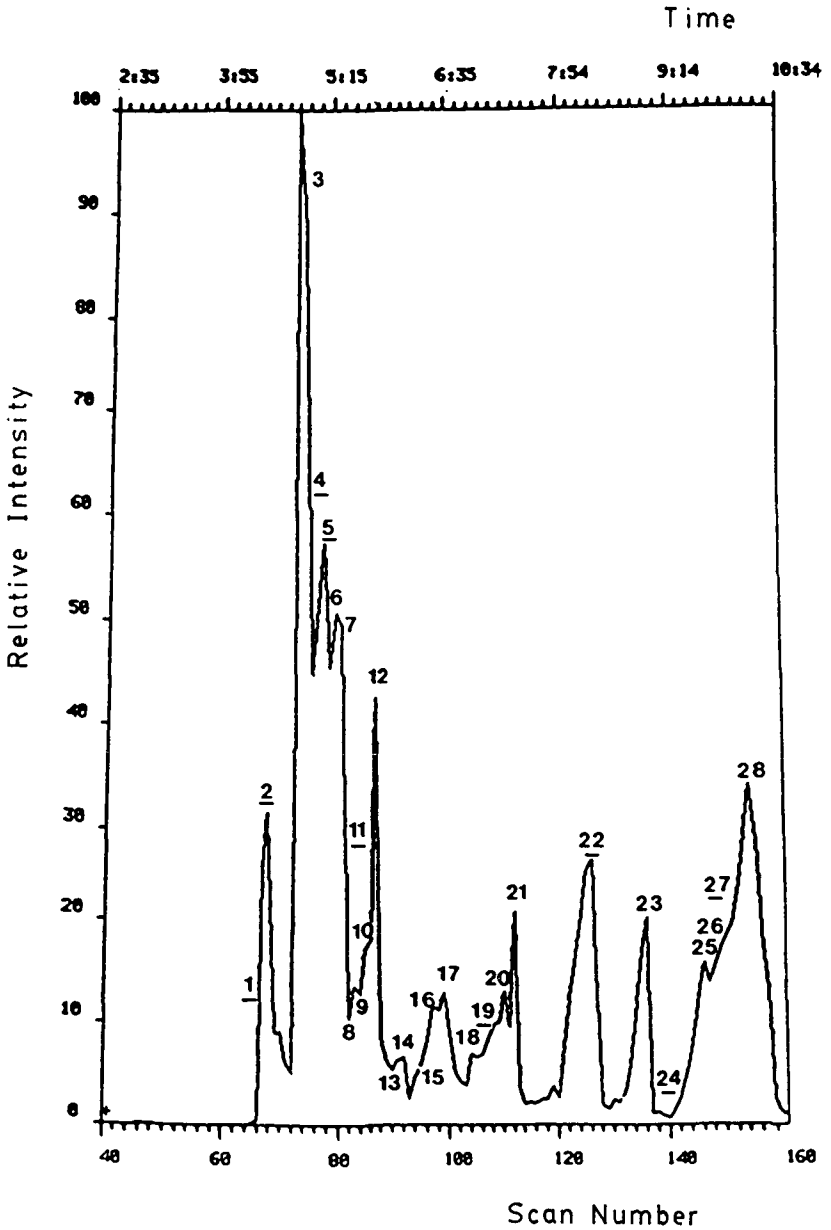

Fig. 4. Expanded 10 min of Fig. 3. Peaks labeled with underlined numbers are sulfur-containing compounds. Time in min:s.

pounds. Based on total sulfur analysis, the total sulfur of a tall oil sample was 470 ppm before purging and 410 ppm after 24 h of purging at 250°C. It is somewhat unexpected that the sulfur content of tall oil did not decrease substantially after prolonged purging at high temperature. This suggests that most sulfur-containing compounds present in

TABLE I

ELEMENTAL COMPOSITION AND EXACT MASSES OBTAINED BY GC–MS

Compound[a]	*Elemental composition*	*Measured mass*	*Deviation (milli-mass units)*[b]	*Compound*[a]	*Elemental composition*	*Measured mass*	*Deviation (milli-mass units)*[b]
1	CH_3SH	48.0033	0.0	60	$C_{11}H_{12}O$	160.0845	−4.7
2	SO_2	63.9593	−2.6	*61*	$C_6H_{12}S$	116.0653	−0.6
3	C_3H_6O	58.0405	−1.4	*62*	$C_7H_{16}S$	132.0957	−1.6
4	CS_2	75.9447	0.5	63	$C_{10}H_{16}O$	152.1239	3.7
5	C_2H_6S	62.0209	1.9	*64*	$C_7H_{14}S$	130.0844	2.8
6	CH_2Cl_2	83.9516	−1.7	65	$C_{13}H_{12}O$	184.0914	2.6
7	C_4H_8O	72.0583	0.8	66	$C_{11}H_{18}$	150.1378	−3.1
8	C_4H_8O	72.0579	0.3	67	$C_{10}H_{14}O$	150.1075	3.0
9	C_4H_6O	70.0430	1.2	*68*	$C_8H_{14}S$	142.0811	−0.5
10	C_4H_8O	72.0569	−0.6	*69*	$C_8H_{16}S$	144.0985	1.3
11	C_2H_6S	62.0209	1.9	*70*	$C_8H_{14}S$	142.0833	1.7
12	C_5H_6O	82.0414	−0.5	*71*	$C_8H_{14}S$	142.0786	−3.0
13	C_6H_{14}	86.1084	−1.2	*72*	$C_7H_{14}S$	130.0837	2.1
14	C_5H_8O	84.0548	−2.7	*73*	$C_{10}H_{18}S$	170.1212	8.1
15	C_6H_{10}	82.0779	−0.3	*74*	$C_8H_{16}S$	144.0987	1.5
16	$C_5H_{10}O$	86.0721	−1.1	75	$C_{19}H_{22}$	250.1699	−2.2
17	C_6H_6	78.0483	1.4	76	$C_{17}H_{30}$	234.2370	2.2
18	C_6H_{10}	82.0803	2.1	78	$C_{17}H_{32}$	236.3510	0.6
19	C_4H_8S	88.0318	−2.9	79	$C_{18}H_{32}$	248.2474	−3.0
20	C_7H_{14}	98.1110	1.4	80	$C_{19}H_{28}$	256.2181	−1.0
21	C_7H_{16}	100.1237	−1.5	81	$C_{19}H_{28}$	256.2185	−0.6
22	$C_2H_6S_2$	93.9906	−0.5	82	$C_{19}H_{28}$	256.2192	0.0
23	C_7H_8	92.0635	0.9	83	$C_{20}H_{32}$	272.2496	−0.8
24	C_5H_6S	98.0146	−4.4	84	$C_{20}H_{32}$	272.2489	−1.5
25	$C_7H_{10}O$	110.0750	1.8	85	$C_{19}H_{28}$	256.2207	1.6
26	C_6H_{10}	82.0776	−0.6	86	$C_{15}H_{14}O$	220.1807	−1.0
27	C_5H_8S	100.0336	−1.1	87	$C_{20}H_{32}$	272.2504	0.0
28	C_8H_{18}	114.1394	−1.4	88	$C_{19}H_{28}$	256.2158	−3.3
29	C_8H_{14}	110.1124	2.8	89	$C_{19}H_{26}$	254.2034	0.0
30	$C_3H_8S_2$	108.0112	4.4	90	$C_{19}H_{26}$	254.2040	0.6

31	C_8H_{10}	106.0765	−1.7	91	$C_{20}H_{32}$	272.2529	2.5
32	C_8H_{10}	106.0811	2.9	92	$C_{20}H_{32}$	272.2465	3.9
33	$C_8H_{12}O$	124.0896	0.8	93	$C_{19}H_{26}$	254.2049	1.5
34	$C_4H_{10}S_2$	122.0225	0.1	94	$C_{20}H_{32}$	272.2507	0.3
35	C_7H_{12}	96.0947	0.8	95	$C_{19}H_{28}$	256.2174	−1.7
36	$C_5H_{10}O$	86.0768	3.0	96	$C_{20}H_{32}$	272.2497	−0.7
37	C_6H_8O	96.0596	2.0	97	$C_{19}H_{28}$	256.2163	−2.8
38	$C_{10}H_{16}$	13.1240	−1.1	98	$C_{20}H_{32}$	272.2480	−2.4
39	C_9H_{12}	120.0921	−1.8	99	$C_{19}H_{26}$	254.2028	−0.8
40	$C_{10}H_{14}$	134.1104	0.9	100	$C_{19}H_{28}$	256.2158	−3.3
41	$C_{10}H_{14}$	136.1266	1.4	101	$C_{19}H_{26}$	254.2013	−2.1
42	$C_9H_{14}O$	138.1031	1.4	102	$C_{20}H_{32}$	272.2504	0.0
43	$C_{10}H_{16}$	136.1242	−0.8	103	$C_{19}H_{28}$	256.2168	−2.3
44	$C_{10}H_{16}$	136.1277	2.5	104	$C_{19}H_{24}$	252.1879	0.1
45	$C_{10}H_{14}$	134.1103	0.7	105	$C_{20}H_{28}$	262.2177	−1.4
46	$C_{10}H_{14}$	134.1088	−0.8	106	$C_{19}H_{26}$	254.2061	2.7
47	$C_{10}H_{16}$	136.1242	−1.0	107	$C_{19}H_{24}$	252.1853	−2.5
48	$C_{10}H_{16}$	136.1277	2.5	108	$C_{19}H_{28}$	256.2205	1.4
49	$C_{10}H_{16}$	136.1225	−2.7	109	$C_{20}H_{28}$	268.2188	−0.3
50	$C_{10}H_{14}$	134.1092	−0.4	110	$C_{20}H_{28}$	268.2214	2.3
51	$C_{10}H_{12}$	132.0931	−0.8	111	$C_{19}H_{26}$	254.2064	3.0
52	$C_{10}H_{14}$	134.1121	2.6	112	$C_{18}H_{28}$	244.2175	−1.6
53	$C_6H_{10}O$	98.0705	−2.7	113	$C_{20}H_{28}$	268.2188	−0.3
54	$C_{10}H_{16}$	136.1257	0.5	114	$C_{20}H_{28}$	268.2166	−2.5
55	$C_{10}H_{16}$	136.1253	0.1	115	$C_{18}H_{32}O$	264.2461	0.8
56	$C_{11}H_{16}$	148.1270	1.8	116	$C_{18}H_{32}O$	264.2451	−0.2
57	$C_7H_{16}S$	132.0986	1.3	117	$C_{16}H_{16}O_2$	240.1167	1.7
58	$C_7H_{14}S$	130.0781	−3.5	118	$C_{20}H_{28}$	268.2190	0.0
59	$C_7H_{16}S$	132.0944	0.5	119	$C_{20}H_{26}$	266.2009	−2.5

[a] Numbers of sulfur-containing compounds are printed in italics.
[b] Deviation = (measured mass − theoretical mass) × 1000.

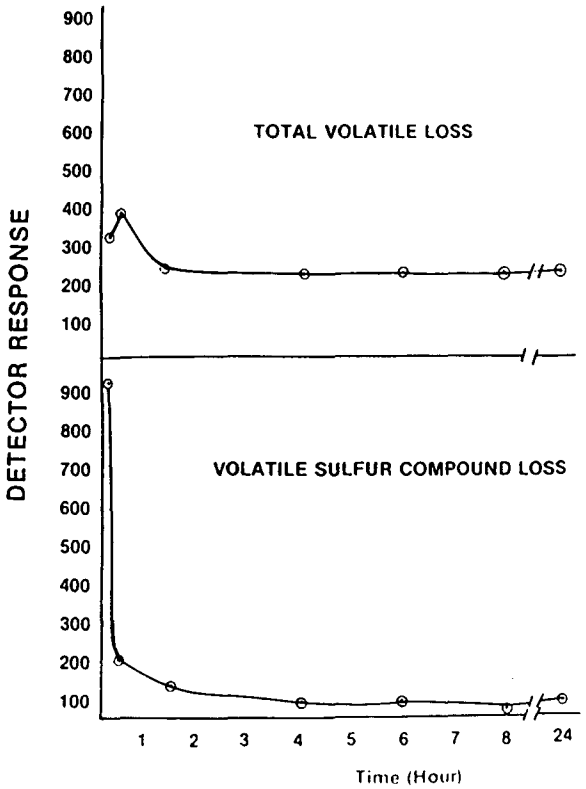

Fig. 5. Loss of total volatiles with time as tall oil was purged with helium at 250°C. The upper figure is for total volatiles measured by FID, the lower figure is for total volatiles measured by FPD.

tall oil have a low volatility. They probably are alkyl mercaptan adducts of fatty acids or rosin acids, which have molecular weights in excess of 300 and boiling points higher than that of tall oil. Prolonged heating could possibly result in scission of the carbon–sulfur bond, producing a sulfur-containing alkyl radical, which could subsequently form some mercaptans.

The continuous conversion of low-volatility sulfur compounds to higher-volatility sulfur compounds at an elevated temperature explains the persistent evolution of traces of sulfur-containing compounds, especially dimethy disulfide, after 24 h of purging at 250°C. Such a conversion is an extremely slow process, particularly at ambient temperature. When a tall oil sample was purged at 250°C for 6 h, then cooled at 20°C, and subsequently purged with helium for 20 min at 20°C, no further release of sulfur compounds was detected.

We also noticed a correlation between the degree of removal of volatile sulfur and the color quality. The crude tall oil has a color of 13–14 on the Gardner color scale. After 1 h of purging with helium at 250°C, the color became 11–12, but continuous purging of up to 24 h did not improve the color beyond that.

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GAS CHROMATOGRAPHIC DETERMINATION OF OXO- AND HYDROXYCARBOXYLIC ACIDS IN SERUM AND URINE OF DIABETIC AND NORMAL SUBJECTS

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SUMMARY

Oxo- and hydroxycarboxylic acids in serum and urine are metabolites of valine, leucine and isoleucine and products of ketogenesis. They are simultaneously determined in the form of their methyl esters and methyl esters–O-methyloximes by gas chromatography, using internal and external standards. Normal values for the urinary excretion of these amino acid metabolites are between 3 ± 2 μmole per 24 h (mean ± standard deviation) for 2-oxoisocaproic acid and 122 ± 58 μmole per 24 h for 3-hydroxyisobutyric acid. In diabetic ketoacidosis the values are increased by a factor of 2–10. In the urine of diabetic patients under fasting conditions all metabolites are elevated and reach a maximum on about the seventh day of fasting. In the serum only 2-oxoisocaproic acid, 3-oxobutyric acid and 3-hydroxybutyric acid reach elevated levels under fasting conditions.

INTRODUCTION

Oxo- and hydroxycarboxylic acids are metabolites of the branched-chain amino acids valine, leucine and isoleucine or products of ketogenesis. In the context of increased amino acid metabolism, their levels in blood serum and urine are elevated during diabetic ketoacidosis. Quantitative data on normal ranges and concentrations in different pathophysiological situations have been incomplete. Enzymatic and especially gas chromatographic (GC)[1–4] and high-performance liquid chromatographic[5,6] procedures have been applied to the determination of some of the metabolites. Oxo- and hydroxycarboxylic acids in diabetes and inborn errors of metabolism have been reviewed[7,8]. In this study, a method has been developed for the simultaneous determination of 2-oxo, 3-oxo, 2-hydroxy- and 3-hydroxycarboxylic acids, representing different pathways of amino acid metabolism and ketogenesis.

EXPERIMENTAL

Samples

Urine samples (24 h) were collected from fifteen healthy individuals (23–63 years old) and fifteen diabetic patients with increased excretion of ketone bodies

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(10–80 years old). From two non-insulin-dependent patients who were fasting for 8 days, 10-ml venous blood samples were drawn on the 2nd, 3rd, 4th, 7th and 8th days. To obtain the serum, the samples were centrifuged at 2000 *g* for 10 min. In addition, 24-h urine samples were taken from four non-insulin-dependent patients, fasting for 8 days, on the 1st, 3rd, 4th, 7th and 8th days. All serum and urine samples were stored at –20°C prior to analysis.

Sample preparation for serum specimens

To 5 ml of serum were added 30 µl of an internal standard solution containing 25 mg of 2-oxocaproic acid, 24 mg of 4-oxobutyric acid, 25 mg of 2-hydroxyvaleric acid and 30 mg of 4-hydroxybutyric acid in 10 ml of distilled water. After mixing the sample with 10 ml of 2-propanol to precipitate the serum proteins, 25 mg of O-methylhydroxylamine hydrochloride were added. The sample was kept at 65°C for 1 h to convert the oxocarboxylic acids into their O-methyloximes. The organic acids were isolated by anion-exchange chromatography on Amberlyst A-26 (Serva, Heidelberg, F.R.G.) and derivatized with diazomethane to form the methyl esters. The methyl esters were prefractionated into four fractions by preparative thin-layer chromatography (TLC), and the fractions were analysed by GC. The details of the method have been described previously[9]. The four fractions were located on the TLC plate by using methyl indolebutyrate as reference substance. With 1.0 as the relative retention value (R_{rel}) for methyl indolebutyrate and 0 for the starting point of the TLC separation, the fractions corresponded to the following zones: fraction 1, R_{rel} = 1.3–1.7; fraction 2, R_{rel} = 1.0–1.3; fraction 3, R_{rel} = 0.7–1.0; and fraction 4, R_{rel} = 0.35–0.7.

Sample preparation for urine specimens

To 30 ml of urine were added 100 µl of the internal standard solution. After mixing the sample with 60 ml of 2-propanol to precipitate any urinary proteins, 150 mg of O-methylhydroxylamine hydrochloride were added and the mixture was processed as described for serum.

Gas chromatographic separation

The GC separations were performed on a Vega 6130 gas chromatograph with a flame ionization detector (Carlo Erba, Hofheim, F.R.G.) under the following conditions: 25 m × 0.25 mm I.D. fused-silica column, coated with OV-1701 (Macherey, Nagel & Co., Düren, F.R.G.); carrier gas, nitrogen at a flow-rate of 4 ml/min; column temperature, 40°C for 10 min, programmed at 2°C/min to 160°C and then at 6°C/min to 230°C, finally held at 230°C for 30 min; injection block temperature, 230°C; sample size, 1 µl at a splitting ratio of 1:10. The gas chromatograph was connected with a CDS-111 integrator (Varian, Darmstadt, F.R.G.).

Identification

The identifications of the oxo- and hydroxycarboxylic acids were based on their methylene units and previous characterizations by GC–mass spectrometry[10]. Mass spectrometric controls were also made when the identification based on methylene units was uncertain. To avoid peak overlapping, samples and hydrocarbon standards (C_5–C_{16}) were analysed separately and in triplicate for the determination of the methylene units.

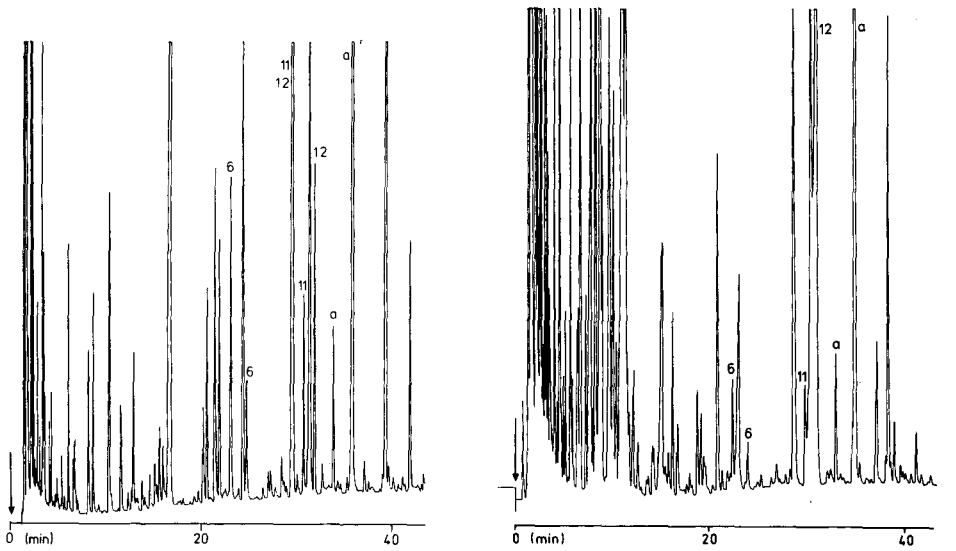

Fig. 1. Gas chromatograms of fraction 1 (early parts) of the organic acids in the urine of diabetic patient A with ketoacidosis (left) and in the serum of diabetic patient B under fasting conditions, 7th day (right). Peaks: 6 = 2-oxoisovaleric acid; 11 = 2-oxo-3-methylvaleric acid; 12 = 2-oxoisocaproic acid; a = internal standard, 2-oxocaproic acid.

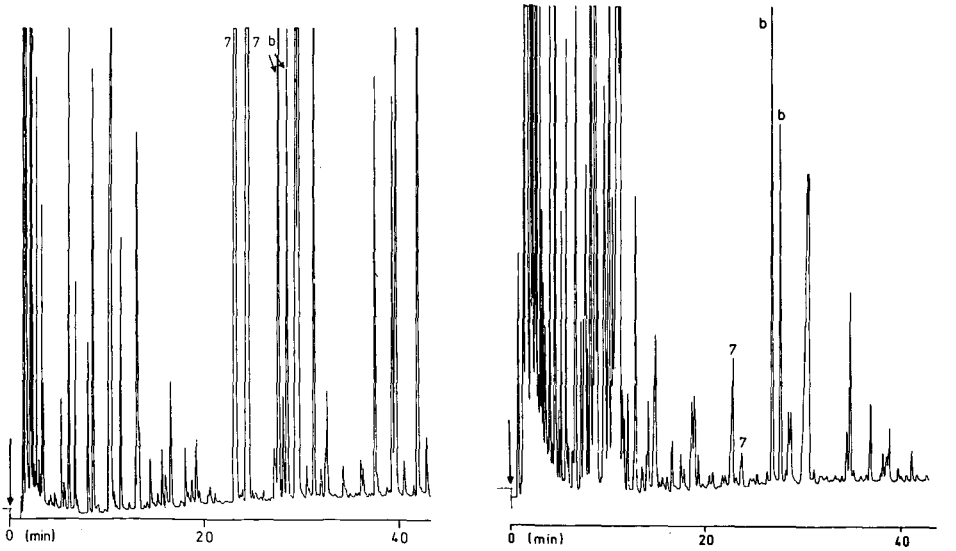

Fig. 2. Gas chromatograms of fraction 2 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 7 = 3-oxobutyric acid; b = internal standard, 4-oxobutyric acid.

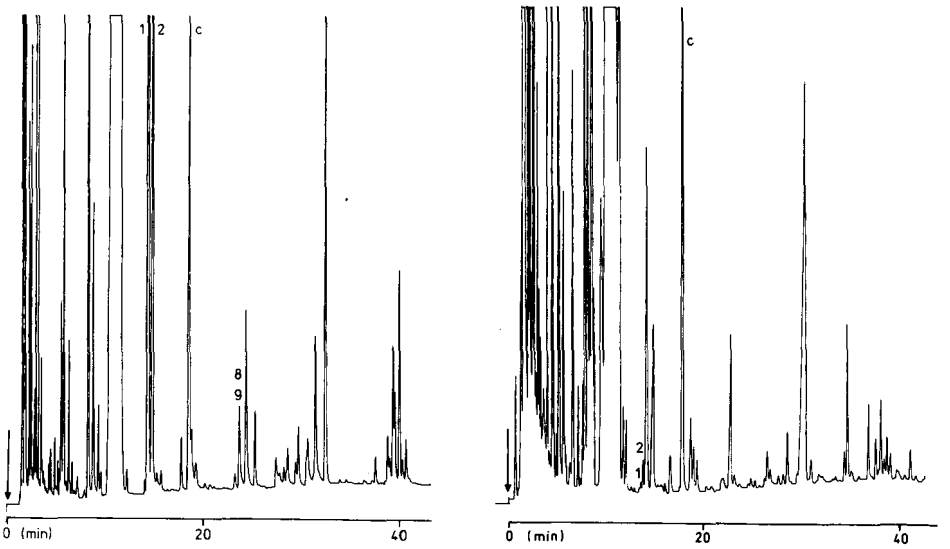

Fig. 3. Gas chromatograms of fraction 3 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 1 = 3-hydroxyisovaleric acid; 2 = 2-hydroxyisovaleric acid; 8 = 2-hydroxy-3-methylvaleric acid; 9 = 2-hydroxyisocaproic acid; c = internal standard, 2-hydroxyvaleric acid.

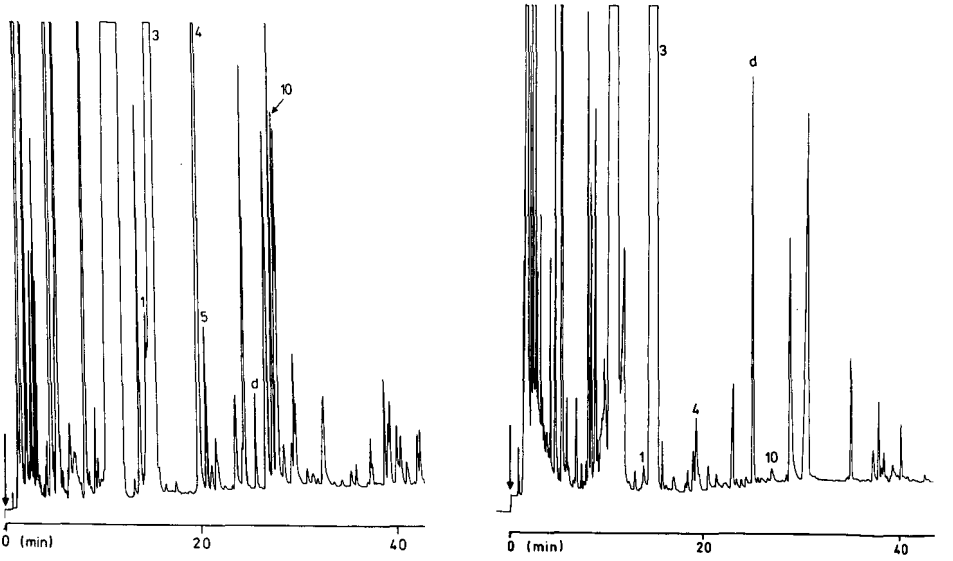

Fig. 4. Gas chromatograms of fraction 4 of the urine sample from patient A (left) and the serum sample from patient B (right). Peaks: 1 = 3-hydroxyisovaleric acid; 3 = 3-hydroxybutyric acid; 4 = 3-hydroxyisobutyric acid; 5 = 3-hydroxy-2-methylbutyric acid; 10 = 3-hydroxy-2-ethylpropionic acid; d = internal standard, 4-hydroxybutyric acid.

Quantitation

Quantitation was based on the ratio of the peak area of the acid to be determined to that of the internal standard and on calibration graphs obtained with four external standard solutions. The standards were prepared by spiking a serum sample with 60, 120, 180 and 240 μg and a urine sample with 120, 240, 360 and 480 μg of each of the reference acids.

RESULTS AND DISCUSSION

Prefractionation

The advantage of prefractionation by TLC, even though it lengthens the sample preparation procedure, is that it helps to avoid interferences in the GC separation and to enrich components present at low concentrations in the biological samples. In the total profile of organic acids, a number of such interferences are observed, which render the quantitation of several low-concentrated oxo- and hydroxycarboxylic acids difficult, especially in pathological samples. For example, after prefractionation, 2-hydroxyisovaleric acid can be measured in samples with large amounts of 3-hydroxybutyric acid and 2-oxoisovaleric acid can be determined in the presence of 3-oxobutyric acid.

With the exception of 3-hydroxyisovaleric acid, which occurs in fractions 3 and 4, all components to be quantitated appear in only one fraction. The distribution of the acids in the four fractions is shown in Figs. 1–4. The oxocarboxylic acids appear in fractions 1 and 2 and the more polar hydroxycarboxylic acids in fractions 3 and 4.

TABLE I

MEAN CONCENTRATIONS OF OXOCARBOXYLIC ACIDS AND HYDROXYCARBOXYLIC ACIDS IN THE URINE OF 15 NORMAL INDIVIDUALS AND 15 DIABETIC PATIENTS WITH KETOACIDOSIS

Compound	*Mean concentration ± S.D. (μmole per 24 h)*	
	Normal individuals	*Diabetics with ketoacidosis*
Valine metabolites:		
2-Oxoisovaleric acid	13 ± 9	35 ± 29
2-Hydroxyisovaleric acid	7 ± 7	252 ± 593
3-Hydroxyisobutyric acid	122 ± 58	691 ± 687
Leucine metabolites:		
2-Oxoisocaproic acid	3 ± 2	16 ± 10
3-Hydroxyisovaleric acid	48 ± 20	101 ± 62
Isoleucine metabolites:		
2-Oxo-3-methylvaleric acid	22 ± 13	48 ± 24
3-Hydroxy-2-methylbutyric acid	13 ± 7	40 ± 30
3-Hydroxy-2-ethylpropionic acid	64 ± 32	258 ± 167
Ketone bodies:		
3-Oxobutyric acid	156 ± 128	6800 ± 5900
3-Hydroxybutyric acid	15 ± 19	2800 ± 2300

The acids to be determined (numbered in Figs. 1–4) are sufficiently well separated from the other organic acids.

Internal standards

The reference substances 2-oxocaproic acid (fraction 1), 4-oxobutyric acid (fraction 2), 2-hydroxyvaleric acid (fraction 3) and 4-hydroxybutyric acid (fraction 4) were chosen as internal standards from among many others tested because they appear in only one fraction, because they are isomers or homologues to the substances to be determined and have similar retention behaviours, because they fit into nearly empty spaces in the complex chromatograms and because they do not occur or occur only in negligible amounts as endogenous compounds, as shown by performing analyses in the absence of the internal standards. This holds true for both the urine and the serum samples (Figs. 1–4).

Separation and quantitation

Except for 2-hydroxy-3-methylvaleric acid and 2-hydroxyisocaproic acid (compounds 8 and 9 in Fig. 3), all hydroxycarboxylic acids are separated. In some instances no baseline separation is achieved. The O-methyloximes of the oxocarboxylic acid methyl esters occur in form of *syn/anti* isomers and are separated into two peaks; for quantitation the peak areas are added. With 2-oxo-3-methylvaleric acid and 2-oxoisocaproic acid, the peaks of the isomeric pairs overlap (Fig. 1). Because it has been found that under the conditions used for sample preparation the ratios of the first and second peaks are relatively constant (2-oxo-3-methylvaleric acid, 10.1 ± 2.2; 2-oxoisocaproic acid, 0.3 ± 0.04), quantitation is based on the two second peaks only.

Working with spiked urine samples, the recoveries for the entire procedure were determined to range between 31% (3-hydroxy-2-methylbutyric acid) and 55% (2-oxoisovaleric acid). On the basis of aqueous solutions with known concentrations (360 and 720 μg/dl) of the reference substances, the accuracy of the method was found to be within ±7–10% (difference from the reference values).

The advantage of the method described over other techniques is that a wide range of metabolites of branched-chain amino acids and the ketone bodies under normal and pathological conditions can be determined simultaneously. The disadvantage is that the procedure is time consuming.

Urinary excretion in normal individuals and diabetic patients with ketoacidosis

For the 2-oxocarboxylic acids, as the primary metabolites of valine, leucine and isoleucine[9], the urinary levels in normal individuals are on average between 3 and 22 μmole per 24 h (Table I). The values for their reduction products are lower by a factor of 2 (*e.g.*, 2-hydroxyisovaleric acid, $\bar{x} = 7$ μmole per 24 h). The main degradation products are the 3-hydroxy metabolites with mean excretion values of up to 122 μmole per 24 h. During diabetic ketoacidosis, there is not only a large increase in the ketone bodies, 3-oxobutyric acid and 3-hydroxybutyric acid in the urine, but also the urinary excretion of all 2-oxo, 2-hydroxy and 3-hydroxy metabolites of the branched-chain amino acids is elevated 2–5-fold. For 2-hydroxyisovaleric acid, the increase is even higher. The individual variation for this metabolite is very large. Among the 3-hydroxy compounds, 3-hydroxyisobutyric acid reaches the highest level ($\bar{x} = 691$

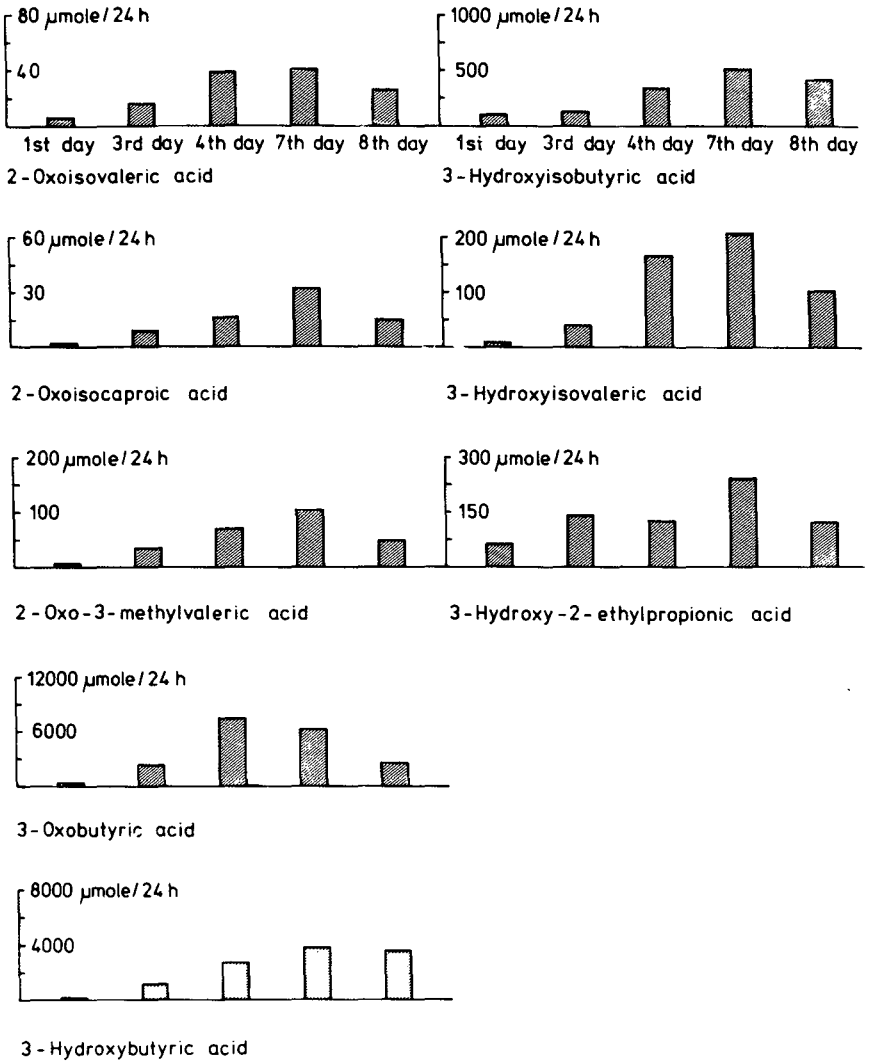

Fig. 5. Oxo- and hydroxycarboxylic acids in the urine of diabetic patients during an 8-day fast period (mean values for four patients).

µmole per 24 h), and as a single substance appears to be the most suitable marker for the amino acid degradation.

Diabetic patients under fasting conditions

In the urine of non-insulin-dependent diabetic patients under fasting conditions, all metabolites of the branched-chain amino acids increase together with ketone bodies (Fig. 5). The maximum is reached on about the 7th day, after which a decrease is generally observed.

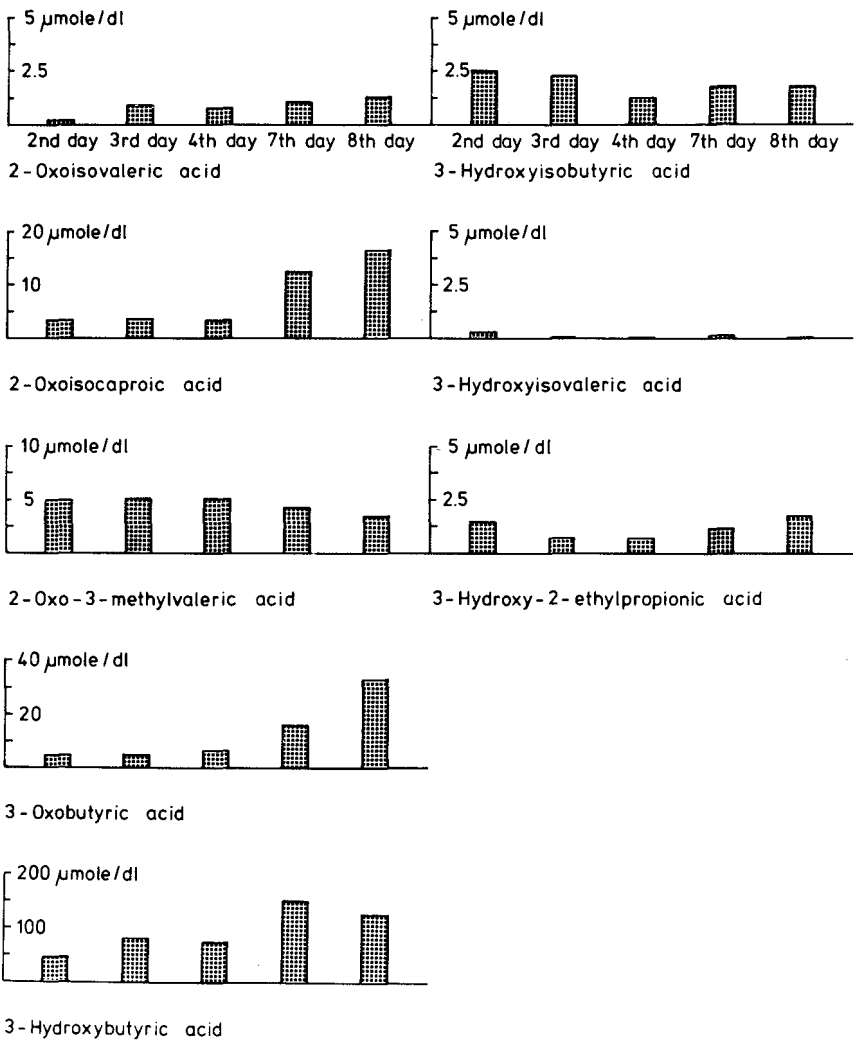

Fig. 6. Oxo- and hydroxycarboxylic acids in the serum of diabetic patients during an 8-day fast period (mean values for two patients).

In the serum an increase is observed only for 2-oxoisocaproic acid and the ketone bodies, 3-oxobutyric acid and 3-hydroxybutyric acid (Fig. 6).

CONCLUSIONS

With the method described, the oxo and hydroxy metabolites of branched-chain amino acids and of the ketone bodies are determined simultaneously and reliably in the normal ranges. The increased amino acid metabolism in conjunction with diabetic ketoacidosis under fasting conditions can be followed in both serum and urine on the basis of several parameters.

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RETENTION MODIFICATION OF NUCLEIC ACID CONSTITUENTS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Secondary equilibria in reversed-phase liquid chromatography have been investigated as a means of enhancing selectivity and optimizing separations of nucleic acid constituents. The retention behavior of various nucleotides, nucleosides and modified compounds has been examined as a function of five different metal ion additives in the mobile phase: K^+, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}. Complexation of the solute molecules with the metal ions changes the electronic structure and alters solute–solvent interactions. Alkali and alkaline earth metals bind primarily to phosphate groups while transition metals also interact with the N^7 of purine bases. All nucleotides were found to be eluted very close to the void volume of the high-performance liquid chromatographic column without any metal additive, but retention increased as the concentration of a given cation increased. The transition metals were found to have the greatest effect, with affinities for nucleotide monophosphates on the order of 100 times greater than potassium, and 10 times that of magnesium. Differences in affinity based upon phosphate structure (*i.e.*, cyclic *vs.* linear), phosphate position (*e.g.*, 2′- *vs.* 3′-monophosphates), and base modification were also noted. The retention of most nucleosides, unlike the charged compounds, remained relatively constant as the ionic strength or type of cation was varied. Also, improvements were obtained in the resolution of some oligonucleotides with the addition of divalent ions to a potassium buffer mobile phase.

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INTRODUCTION

The basic parameters that affect retention behavior in reversed-phase high-performance liquid chromatography (HPLC) (*i.e.*, pH, type and amount of organic modifier, ionic strength, and temperature) have been investigated in detail for nucleotides, nucleosides, and bases[1–6]. Since these compounds are ionizable, some secondary equilibria effects using oppositely charged mobile phase additives have also been examined[1,7–11]. In this case, pairing reagents, such as tetraalkylammonium or alkylsulfonate ions may be added, resulting in one type of interaction, or small, charged inorganic constituents may be used for other types of interactions. It is assumed that large hydrophobic ions are adsorbed on the non-polar stationary phase and that ion-pair formation then occurs at the mobile phase–stationary phase interface[8,12]. With the small inorganic ions the pairing interaction occurs primarily in the mobile phase, although the exact retention mechanism may depend upon the actual operating conditions[1,9,12].

Nucleotides are known to complex with metal ions, alkali and alkaline earth metals binding to the phosphate groups, and transition metals also interacting with the N^7 of the purine bases[1,11,13–15]. This complexation changes the electronic structure and the conformation of the compounds, and this in turn alters solute–solvent interaction and retention behavior. Horváth *et al.*[9] demonstrated that the addition of Mg or Zn ions to the mobile phase resulted in increased retention of some nucleoside di- and triphosphates. The chromatographic data showed a dependence of the capacity factors on the metal ion concentration and allowed the determination of apparent stability constants. The increase in capacity factors was attributed to the reduction of electronic charge in the molecules, allowing for stronger interactions on the non-polar stationary phase. Cohen and Grushka[10] also examined the effects of some divalent metal ions on the retention of nucleotides, nucleosides, and bases. Ni and Zn at low levels reduced k' values for nucleotides, while higher concentrations slightly increased the values. Mg at all levels was found to decrease retention while Cu provided an increase. Braumann and Jastorff[7] on the other hand demonstrated that Mg and K increased the retention of some nucleoside mono- and cyclicphosphates. Their data also indicate that differences in complexation (and retention) may be related to the structure of the phosphate group and to the nature of the cation. The discrepancies in data in these studies likely pertain to differences in buffer composition and ionic strengths[1,7]. The retention will be influenced by all the cations present in the mobile phase and if the concentration of the metal ion under investigation is low relative to other ions, the effect may appear negligible.

In this study, the effects of five different metal ions (K^+, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+}) on the retention behavior of a variety of nucleotides and nucleosides differing in base modification, phosphate structure, or phosphate position have been examined. Mobile phases containing only the one cation under investigation were used to eliminate the confounding effects other metal ions may have. The data were generated to indicate how metal ion interaction may be utilized to enhance selectivity and optimize separations. Results for some oligonucleotides are also reported.

EXPERIMENTAL

Instrumentation

The oligonucleotides were analyzed on a Waters liquid chromatographic system, equipped with a Model 600E multisolvent delivery system, a Model U6K injector, and a Model 490 programmable multiwavelength detector (Waters Chromatography Division, Millipore, Millford, MA, U.S.A.). Data acquisition was performed by a Maxima 820 chromatography workstation (Dynamic Solutions, Millipore, Ventura, CA, U.S.A.). The other compounds were analyzed on a Beckman LC system consisting of two Model 112 solvent delivery modules and a Model 421 system controller (Beckman Instruments, Altex Division, San Ramon, CA, U.S.A.). Injections were made with a Rheodyne Model 7125 injector, using a 20-μl sample loop (Rheodyne, Cocatati, CA, U.S.A.). The UV absorbance was monitored at 254 nm.

Materials and methods

The oligonucleotides were separated on an Alltech 250 × 4.6 mm C_{18} HS column (7 μm), while other nucleic acid constituents were analyzed on a Vydac 250 × 4.6 mm C_{18} HS column (10 μm), (both from Alltech Assoc., Deerfield, IL, U.S.A.). Potassium dihydrogenphosphate or chloride salts of Mg, Mn, Zn and Ni were used for preparing the mobile phase. The pH of these solutions was adjusted to 6.6, using Tris–HCl or Tris, with the exception of the KH_2PO_4 solution for which KOH was used. Methanol (Burdick & Jackson, Muskegon, MI, U.S.A.) was then added to provide a 20% (v/v) concentration and the final solutions were filtered through 0.45-μm Nylon 66 membranes (Supelco, Bellefonte, PA, U.S.A.). Data were obtained at a flow-rate of 1.5 ml/min at ambient temperatures. Retention times were measured from the start of the injection and all analyses were performed in duplicate.

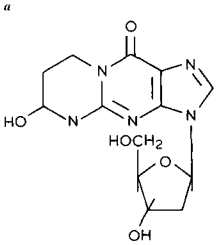
KH_2PO_4 was obtained from Mallinckrodt (St. Louis, MO, U.S.A.), $MgCl_2$ from J. T. Baker (Phillipsburg, NJ, U.S.A.), $MnCl_2$ and $NiCl_2$ from Tridom/Fluka (Hauppauge, NY, U.S.A.), Tris–HCl and Tris from Eastman Kodak (Rochester, NY, U.S.A.), KOH from Fisher Scientific (Pittsburgh, PA, U.S.A.), and $ZnCl_2$ from Aldrich (Milwaukee, WI, U.S.A.). Only certified or analyzed purity grade reagents were used. Over 35 different nucleic acid constituents were examined for changes in retention behavior as a function of metal ion additive (type and concentration) to the mobile phase. These compounds are listed in Table I. They were obtained from Schwarz Labs. (Orangeburg, NY, U.S.A.), Aldrich, Sigma (St. Louis, MO, U.S.A.), P-L Biochemicals (Milwaukee, WI, U.S.A.), Calbiochem (Los Angeles, CA, U.S.A.), or Tridom/Fluka. The compounds differed in their total charge (*e.g.*, deoxyadenosine diphosphate, deoxyadenosine monophosphate and deoxyadenosine), the location of charge (*e.g.*, guanosine 2′-monophosphate and guanosine 3′-monophosphate), or in the structure of the base (*e.g.*, 8-bromoadenosine 5′-monophosphate and adenosine 5′-monophosphate). The oligonucleotides were purchased from Pharmacia (Piscataway, NJ, U.S.A.). In addition to the commercially available compounds, two deoxyguanosine derivatives (6- and 8-hydroxyacrolein–deoxyguanosine adducts), synthesized according to the procedure by Chung *et al.*[16], were examined. Stock solutions (10 m*M*) of all the standards were prepared and diluted 1:50 for working standards, using HPLC-grade water (Burdick & Jackson).

TABLE I

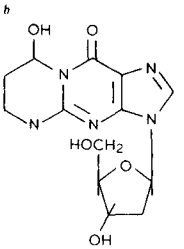
NUCLEIC ACID CONSTITUENTS EXAMINED AS A FUNCTION OF METAL ION ADDITIVE

Compound	*Abbreviation*
Adenosine 5′-monophosphate	5′-AMP
Adenosine 3′-monophosphate	3′-AMP
Adenosine 2′-monophosphate	2′-AMP
8-Bromoadenosine 5′-monophosphate	8-Br-5′-AMP
Adenosine 2′,3′-monophosphate	2′,3′-cAMP
Adenosine 3′,5′-monophosphate	3′,5′-cAMP
8-Bromoadenosine 3′,5′-monophosphate	8-Br-3′,5′-cAMP
Guanosine 2′-monophosphate	2′-GMP
Guanosine 3′-monophosphate	3′-GMP
Guanosine 3′,5′-monophosphate	3′,5′-cGMP
Guanosine 2′,3′-monophosphate	2′,3′-cGMP
2′-Deoxycytidine 5′-monophosphate	dC-5′-MP
2′-Deoxycytidine 3′-monophosphate	dC-3′-MP
2′-Deoxycytidine 3′,5′-diphosphate	dC-3′,5′-DP
Cytidine 5′-monophosphate	5′-CMP
2′-Deoxyadenosine 5′-monophosphate	dA-5′-MP
2′-Deoxyadenosine 5′-diphosphate	dADP
2′-Deoxyadenosine	2′-dA
2′-Deoxycytidine	2′-dC
5-Chloro 2′-deoxycytidine	5-Cl-2′-dC
5-Methyl 2′-deoxycytidine	5-Me-2′-dC
2′-Deoxyuridine	2′-dU
5-Bromo 2′-deoxyuridine	5-Br-2′-dU
2′-Deoxyguanosine	2′-dG
8-Bromodeoxyguanosine	8-BrdG
6-Hydroxyacrolein deoxyguanosine adduct[a]	6-OHAdG
8-Hydroxyacrolein deoxyguanosine adduct[b]	8-OHAdG
Adenosine	A
Guanosine	G
8-Bromoguanosine	8-BrG
Thymidine	T
3-Methylthymidine	3-MeT
Uridine	U
5-Aminouridine	5NHU-s
5-Aminouracil	5NHU
Cytidine	C

a



b



RESULTS

Data for all compounds were obtained without the addition of any metal ions, using only 20% aq. methanol as the mobile phase. Under these conditions, most charged compounds were found to be eluted close to the void time of the column. However, as metal ions were added, the retention times were found to increase significantly. Fig. 1 shows results for various adenosine nucleotides as the K^+ concentration is increased from 1 to 100 m*M*. The two cyclic compounds, adenosine 3′,5′-monophosphate and adenosine 2′,3′-monophosphate, and deoxyadenosine diphosphate show an almost linear increase over the entire range. The measurable increases between no additive and 1 m*M* K^+ indicate that these compounds have a strong affinity for the metal ion. Increases in retention for adenosine 3′-monophosphate, adenosine 5′-monophosphate, and deoxyadenosine 5′-monophosphate on the other hand are more pronounced above 1 m*M* K^+. Overall, the singly charged cyclic nucleotides showed the greatest change in retention, followed by the doubly charged compounds, and finally deoxyadenosine diphosphate. These results are in agreement with the work of Braumann and Jastorff[7], who also suggested that the resulting cyclic nucleotide–metal complexes may attain electrical neutrality, as

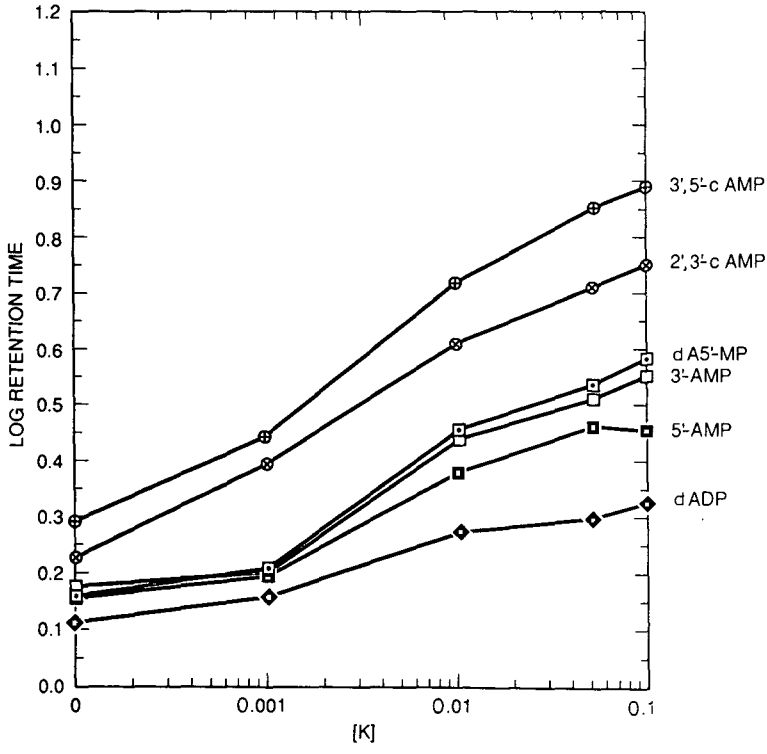


Fig. 1. Retention behavior of various adenosine nucleotides as a function of K^+ levels in mobile phases containing different molar concentrations of KH_2PO_4 in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

evidenced by their capacity factors, which are similar to uncharged adenosine (see Fig. 6).

The effect of magnesium ion on charged nucleotides was more marked, as indicated by the increases in retention at low cation concentration (*i.e.*, 1 m*M* Mg^{2+}). Since magnesium has a higher affinity for phosphate[7], these results may be expected. From Fig. 2 it can also be concluded that retention behavior is not significantly changed above 10 m*M* Mg^{2+}, and this indicates that the effects of complexation are saturated at that level. Results for the other nucleotides (*i.e.*, GMP and dCMP compounds) were similar to the AMP compounds, with retention increasing up to about 10 m*M* Mg^{2+} and then leveling off. However, there was no preferential binding of the Mg to the guanosine cyclic monophosphates over 2'-GMP or 3'-GMP. The retention of all four GMP compounds was about the same at each Mg level. Both the GMP and dCMP nucleotides had capacity factors approaching their corresponding nucleosides at cation saturation (*i.e.*, 10 m*M* Mg^{2+} or above). From the data in Fig. 2 it is also interesting to note that the base-modified 8-Br-3',5'-cAMP and 8-Br-5'-AMP exhibited sufficiently different retention behavior from their corresponding parent compounds (*i.e.*, 3',5'-cAMP and 5'-AMP) with the addition of Mg ion to the mobile phase that they may be resolved. At 0.1 *M* Mg^{2+} the resolution for 8-Br-3',5'-cAMP and 3',5'-cAMP is 5.6 and for 8-Br-5'-AMP and 5'-AMP is 0.9 ($R = 2\Delta t/W_{b1} + W_{b2}$, where t = retention time and W_b = base width of the peak).

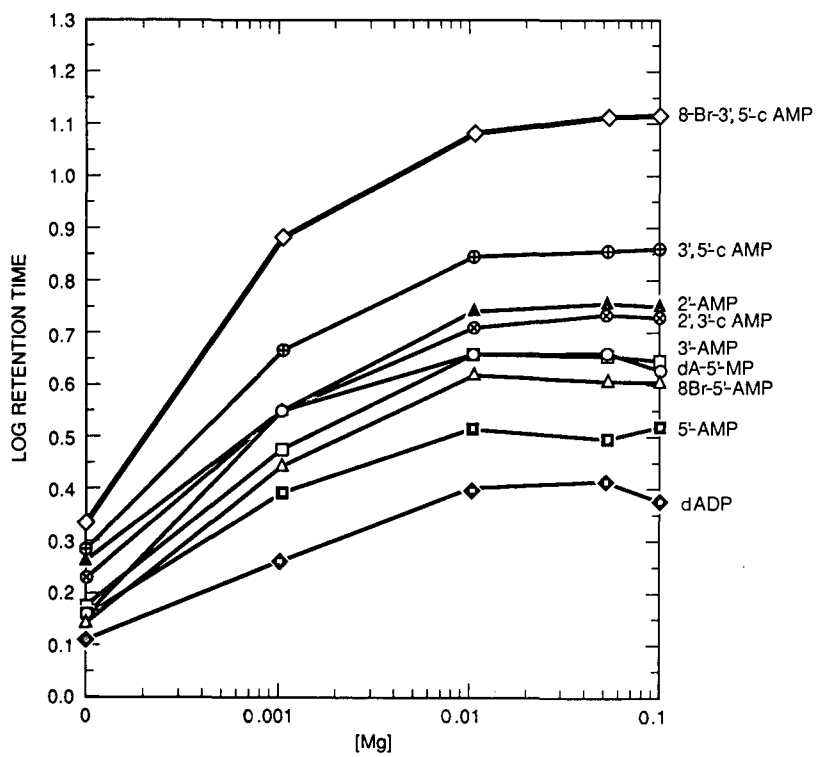


Fig. 2. Retention of adenosine nucleotides as a function of Mg^{2+} levels in mobile phases containing different molar concentrations of $MgCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

Qualitatively, the results for Mn were similar to those for Mg. The major difference is that Mn appears to have a higher affinity for the nucleotides (*i.e.*, the capacity factors for most compounds at 1 m*M* Mn were similar to those at 10 m*M* Mg). Ni and Zn were also found to have very high affinities for the charged compounds. Data for adenosine mononucleotides are shown in Figs. 3 and 4. It should be noted that nucleotides carrying a negative charge of 2 or more were not eluted from the column with 5 m*M* or 10 m*M* Zn^{2+} or with 10 m*M* Ni^{2+} ion added. To determine whether these compounds may be precipitating on the column, 100-ppm solutions of the standards in the mobile phase (10 m*M* $ZnCl_2$ or $NiCl_2$ in 20% aq. methanol) were prepared and examined for any cloudiness. Deoxyadenosine diphosphate, 3',5'-dCDP, 2'-GMP, 3'-GMP, 5'-dCMP, and 3'-dCMP were found to precipitate with the $ZnCl_2$ and 2'-GMP and 3'-GMP with the $NiCl_2$ solution. After the analytical column had been exposed to 10 m*M* Zn^{2+} for several days and after the analysis of various nucleotides had been attempted, the retention factors were also found to fluctuate for both charged and uncharged compounds. As efforts to reactivate the column by flushing it with water were unsuccessful, the column was replaced. The new column was characterized by analyzing all the standards with the 10 m*M* $MgCl_2$ mobile phase, and the capacity factors were found to be within 5% of the old column. Studies with Ni and Zn cations then proceeded, starting with 0.5 m*M* concentrations and carefully washing the column before introducing the next higher concentration. Compounds

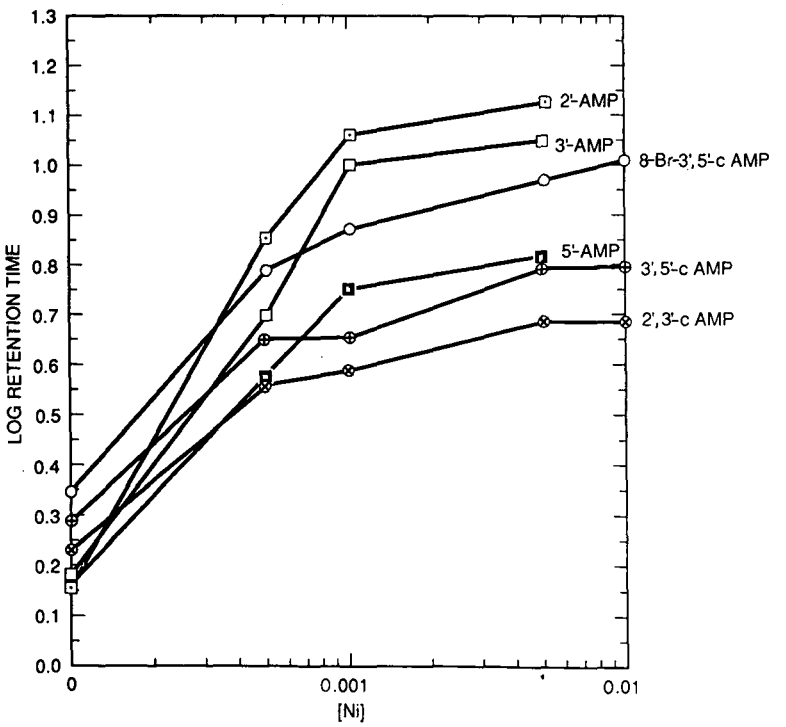


Fig. 3. Retention of adenosine nucleotides as a function of Ni^{2+} levels in mobile phases containing different molar concentrations of $NiCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

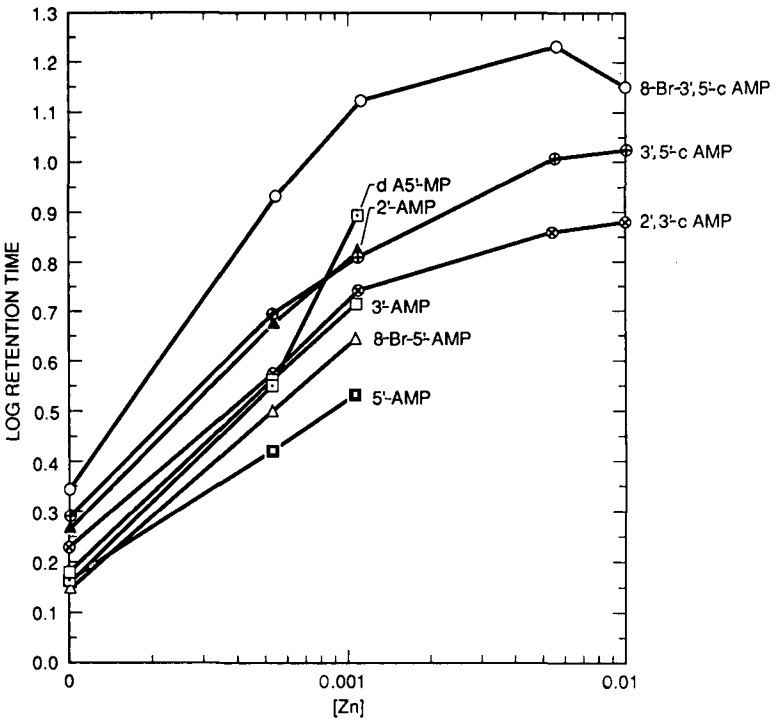


Fig. 4. Retention of adenosine nucleotides as a function of Zn^{2+} levels in mobile phases containing different molar concentrations of $ZnCl_2$ in 20% (v/v) aq. methanol. Abbreviations identified in Table I.

that were not eluted from the old column at a given cation concentration were not reexamined on the new column under the same conditions. Comparing the Ni, Mn and Zn data, Ni was shown to have a much stronger affinity for the monophosphates than for the cyclic monophosphates. In fact, differences in retention between 2'- or 3'-GMP and 2',3'-cGMP or 3',5'-cGMP at 1 m*M* Ni^{2+} exceeded 10 min.

Unlike the charged species, the retention times of most uncharged compounds remained fairly constant as the concentration or the type of metal ion was varied. Fig. 5, which shows the results of various nucleosides with Ni^{2+} ion, is representative of the data obtained for these compounds with K^+, Mg^{2+}, Mn^{2+}, or Zn^{2+}. The only compound found to shift retention as a function of the type of metal ion added was 8-aminoguanosine. With 10 m*M* Zn^{2+} the retention time more than doubled from that obtained using 10 m*M* of any of the other metal cations. Fig. 6 shows data for this compound and other purine nucleosides as a function of the Zn^{2+} concentration. The addition of the amine group to guanosine results in a compound that may have multiple conjugated forms of varying stability. At different ionic strengths forms that are more or less amenable to complexation or aggregation may predominate, leading to differences in retention. The reason for the increase in retention of this compound with Zn^{2+} only is not understood at this time. It is also interesting to note that the 6- and 8-hydroxyacrolein derivatives of deoxyguanosine exhibited different retention

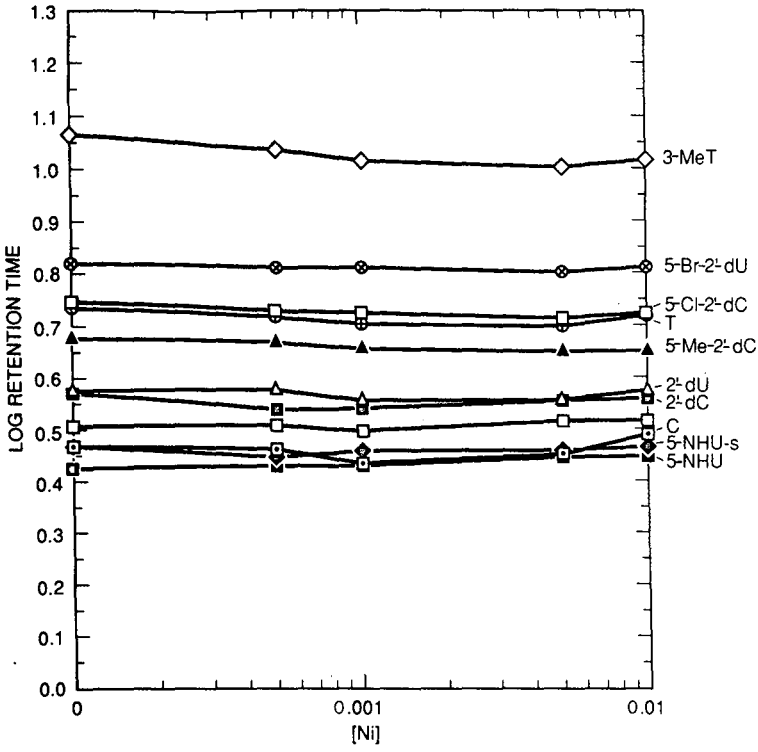


Fig. 5. Retention of various nucleosides and modified nucleosides as a function of Ni^{2+} concentration in the mobile phase. Abbreviations identified in Table I.

properties, despite similarities in their structure. The retention between the two isomers at 10 m*M* Zn^{2+} is 1.7.

The effects of metal cations on the separation of some oligonucleotides were also briefly investigated. A mixture of tetramers containing 5'-d(GGCC)-3', 5'-d(CCGG)-3', 5'-d(CGCG)-3', 5'-d(GCGC)-3', and 5'-d(AGCT)-3' was examined, using 0.01, 0.1, and 0.2 *M* KH_2PO_4 at pH 6.6 with 20% (v/v) aq. methanol (see Fig. 7). At 10 m*M* K^+ only d(AGCT) was separated from the remaining compounds. However, as the K^+ ion concentration increased, increasing complexation resulted in greater differences in retention among the five compounds. The best isocratic separation was thus obtained with 0.2 *M* K^+, where four peaks and a shoulder were evident. Methanol gradient conditions which would optimize the separations at 0.1 *M* and 0.2 *M* K^+, were also examined, with the results shown in Fig. 8. Attempts to resolve these compounds by using only divalent metal ions were unsuccessful, since the oligonucleotides were found to be eluted as broad, tailing peaks. Peak asymmetry and tailing have been reported as the most significant problem in ion-pair HPLC, and have been attributed to association and dissociation processes[12]. However, we determined that the addition of divalent ions to the K^+ buffer would improve the overall resolution and maintain the efficiency. Figs. 9 and 10 show separations of the tetramers and hexamers, respectively, when KH_2PO_4 doped with $NiCl_2$, was used as the mobile phase. In the case of the

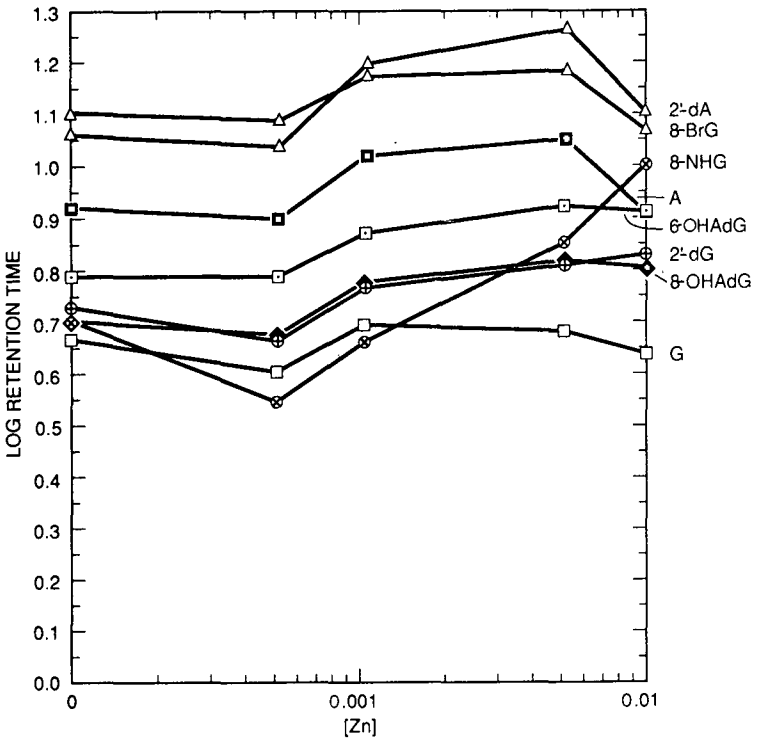


Fig. 6. Retention of nucleosides and modified nucleosides as a function of Zn^{2+} concentration in the mobile phase. Abbreviations identified in Table I.

hexamers, increasing the Ni^{2+} concentration from 1.2 to 3 m*M* altered the selectivity in such a way that 5′-d(ITGCAT)-3′, and 5′-d(ATG5MeCAT)-3′ were found to be resolved. When only K^+ buffer was used under similar gradient conditions, only four peaks were observed for the hexamers, in contrast to the six peaks observed when Ni^{2+} was added.

DISCUSSION

This study was undertaken to determine how secondary equilibria through metal-ion complexation may be used to control selectivity and optimize separations of various nucleic acid constituents. Other investigators have examined cation binding for the purpose of determining stability constants or relating chromatographic behavior to known physico-chemical properties of the complex[7,9,10]. The data in the literature also appear to be conflicting, both increasing and decreasing retention with the addition of a given metal ion to the mobile phase having been reported[7,10]. We have shown that as the concentration of the cation is increased, the retention of nucleotides also increases up to the level where the effects of complexation are saturated.

The transition metals were found to have a *ca.* ten times greater affinity for the

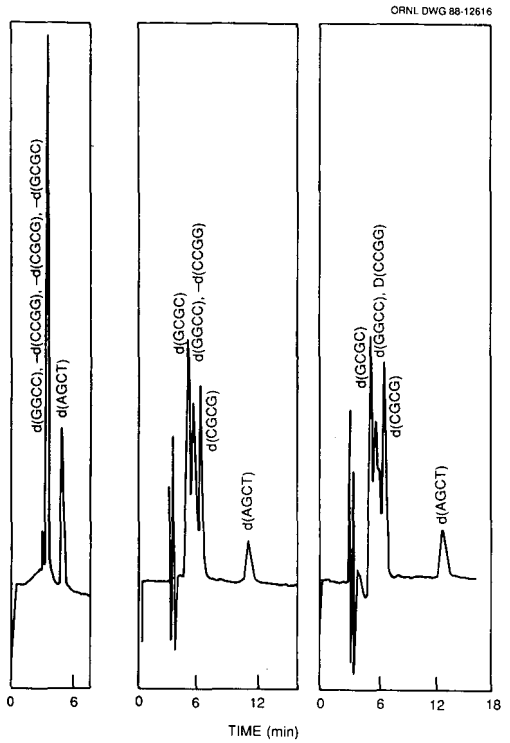


Fig. 7. Isocratic separation of tetramers with (a) 0.01 *M*, (b) 0.1 *M*, and (c) 0.2 *M* KH_2PO_4 (pH 6.6) in 20% (v/v) aq. methanol at a flow-rate of 1 ml/min.

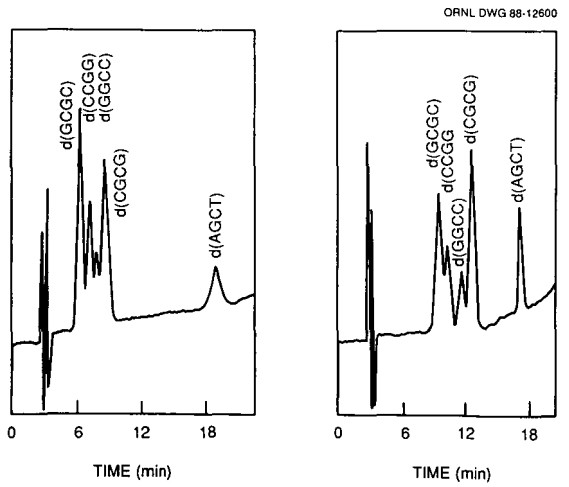


Fig. 8. Gradient separation of five tetramers. (a) Eluent A, 0.1 *M* K H_2PO_4 (pH 6.6) in 10% aq. methanol; eluent B, 0.1 *M* KH_2PO_4 (pH 6.6) in 20% aq. methanol; linear program at 5 min from 80 to 100% B in a 10-min interval; flow-rate 1.0 ml/min. (b) Eluent A, 0.2 *M* KH_2PO_4 (pH 6.6) in 10% aq. methanol; eluent B, 0.2 *M* KH_2PO_4 (pH 6.6) in 30% aq. methanol; linear program at 6 min from 35 to 90% B in a 10-min interval; flow-rate, 1.0 ml/min.

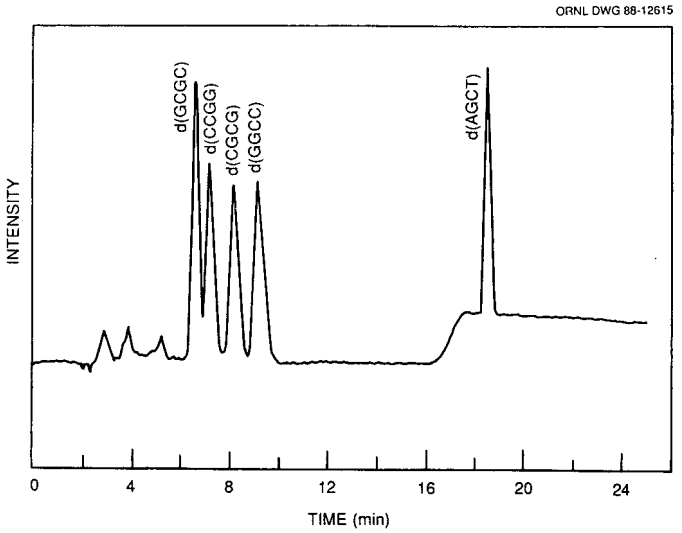


Fig. 9. Separation of five tetramers with the addition of $NiCl_2$ to the mobile phase. Eluent: 0.14 *M* KH_2PO_4, pH 6.6, 1.2 m*M* $NiCl_2$, 17% aq. methanol increasing to 25% aq. methanol after 5 min; flow-rate 1.0 ml/min.

nucleotides than magnesium and 100 times that of potassium, as determined by the differences in concentration at which the effects become saturated. These results on relative affinities follow the order of reported stability constants for nucleotides (*i.e.*, $K^+ < Mg^{2+} < Mn^{2+} < Ni^{2+} < Zn^{2+}$) in coordinating ability[15]. Differences in retention behavior, based upon total charge, charge position, and base modification, were also noted.

Although the divalent metal ions significantly enhanced the separation of charged compounds, some peak broadening for nucleoside monophosphates (linear as

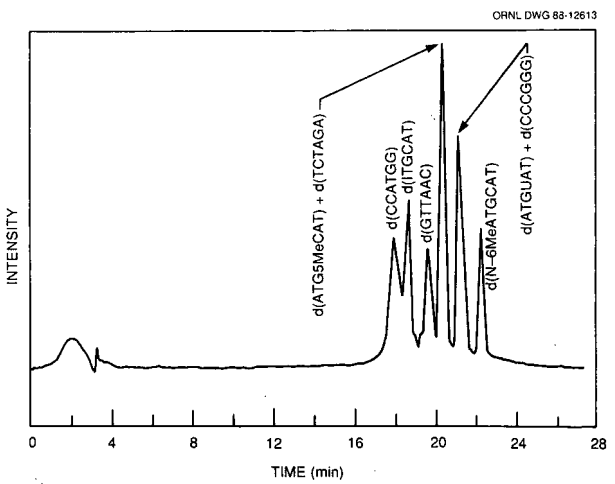


Fig. 10. Separation of hexamers. Eluent: 0.14 *M* KH_2PO_4 (pH 6.6) 1.2 m*M* $NiCl_2$, in 18% aq. methanol increasing to 28% aq. methanol after 5 min; flow-rate 1.0 ml/min.

opposed to cyclic) was observed when Ni^{2+} or Zn^{2+} was used. More importantly, the column performance was found to degenerate after relatively high levels of these ions had been introduced. This may have been due to the precipitation of some complexes in the column or to an exchange of ionic sites, leading to the incorporation of the metal cations. Horváth *et al.*[9] have also reported that Zn^{2+} at relatively high levels (20 m*M*) irreversibly changed the nature of a reversed-phase column they were working with. They reasoned that the changes may have resulted from interaction of the metal ion with surface silanols in the packing material. However, lower concentrations may not pose a problem in terms of column degeneration. Since the separations attainable for the mononucleotides may be effected at low concentrations with these ions, there may be no need to use higher levels. For example, the resolution of guanosine monophosphates from guanosine cyclic monophosphates at 1 m*M* Ni^{2+} may be the same as at 5 m*M* Ni^{2+}.

The nucleosides were largely unaffected by the type or concentration of metal additives. This was the case for both purine- and pyrimidine-type compounds. Notably, however, it was determined that the resolution of oligonucleotides may be improved if low concentrations of divalent cations are added to a K^+ mobile phase. Since complexation occurs mainly in the aqueous phase, rapid rates of formation and dissociation of the complex should result in sharp, symmerical peaks[12]. This is observed when only K^+, or K^+ doped with other metals, is used, but not when only divalent ions are used. In the latter case, the metal ion may complex with more than one phosphate group in the oligonucleotide chain, leading to prolonged interactions, in contrast to K^+, which may couple to only one phosphate at any given time.

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ANALYSIS OF *IN SITU* METHYLATED MICROBIAL FATTY ACID CONSTITUENTS BY CURIE-POINT PYROLYSIS–GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A newly developed Curie-point pyrolyzer has been used to analyze microbial fatty acid constituents. The pyrolysis was carried out on ferromagnetic wires, which were loaded into the gas chromatographic injector–pyrolyzer via a retractable Pyrex glass tube. Fatty acid methyl esters were obtained by *in situ* methylation of the free and glycerol bound fatty acids with trimethylanilinium hydroxide. The procedure was applied sucessfully to whole microorganisms. There was good agreement between the fatty acid methyl ester profiles from the whole-cell pyrolysis and from the lipid extracts of the corresponding organisms.

INTRODUCTION

Pyrolysis (Py)–gas chromatography (GC) and Py–GC–mass spectrometry (MS) have developed into powerful techniques for the analysis of non-volatile synthetic polymers and complex biological materials[1]. Thermal degradation of these substances generate a large variety of decomposition products, which range from chemically related isomers to compounds that differ in polarity, structure and molecular mass. Such a multitude of fragments is desirable if chromatographic profiles are used to characterize biopolymers or whole microorganisms; however, the diverse chemical nature of the decomposition products demands rigid standards for the analytical system.

Characterization of microorganisms by pyrolysis profiles requires strict control of factors such as culture medium, pH, physiological age of cells, temperature, isolation technique and pyrolysis conditions[2]. However, even standardized procedures often fail to maintain good reproducibility of the pyrolysis patterns of whole microorganisms. Better results have been obtained when the characterization was based on the composition of cellular components, such as the cell wall fraction or membrane constituents. Pyrolysis patterns of these cellular subfractions provide more

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definitive characteristic information for taxonomic comparison. However, the problem with this approach is that the sample preparation prior to analysis is quite extensive.

One of the most popular signature components of microorganisms are cellular fatty acids[3,4]. Fatty acid methyl ester profiles have been used successfully in the classification of microorganisms[4,5], in ecological studies aimed at defining microbial community structures[6], in the typing of depositional environments[7], and as specific markers in tracing the origin of organic materials in deposits[8,9]. The analysis of fatty acids is generally preceded by a sample preparation, which includes the extraction of the lipids with organic solvents, followed by the hydrolysis of the saponifiable glycerides. The free fatty acids are then converted into their corresponding methyl esters[10]. The volatile nature of these esters allows the direct analysis by GC.

The extensive sample preparation necessary for this type of analysis, led us to investigate the release of cellular fatty acids from whole microorganisms by Py–GC techniques. Since chromatographic separation of fatty acid methyl esters is preferred over the chromatography of free acids, a number of methylating reagents for *in situ* derivatization of free and glycerol-bound fatty acids during Py–GC were investigated. We report here the use of trimethylanilinium hydroxide[11] as an additive to whole cells prior to pyrolysis.

A Curie-point pyrolysis unit was selected over the conventional, filament type pyrolyzer. The unit has a low dead volume, which is important for maintaining a high concentration of the derivatizing agent during pyrolysis and ensures a fast removal of the reaction products on to the chromatographic column. The disposable ferromagnetic filaments used in this type of pyrolyzer, are easily loaded with sample and can be heated to final temperatures ranging from 360 to 770°C in less than 0.5 s. Optimum conditions for sample derivatization and transfer on to the column can be fine-tuned by split flow adjustment.

EXPERIMENTAL

Lipid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and from Supelco (Supelco Park, Bellefonte, PA, U.S.A). A 1% solution of trimethylanilinium hydroxide (TMAH) in methanol, (Eastman-Kodak, Rochester, NY, U.S.A.) was used as the reagent for *in situ* methylation. The following microorganisms were grown on tryptic soy agar (Difco Lab., Detroit, MI, U.S.A.) at 37°C: *Escherichia coli*, *Enterobacter aerogenes*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Pseudomonas maltophilia* and *Staphylococcus aureus*. Cells were harvested after 48 h by scraping the cells off the agar surface with an inoculating loop. Fatty acid extracts of the microrganisms were prepared according to the procedures described by Moss[3] and Miller[12].

A schematic diagram of the Curie-point pyrolysis apparatus is shown in Fig. 1. The system, which is similar to the prototype used in a study by Meuzelaar *et al.*[13], was provided by Somatogenetics Instruments (Broomfield, CO, U.S.A.). It is equipped with a 75 W/400 kHz radiofrequency (RF) power supply. The samples were deposited on disposable ferromagnetic filaments that have Curie points ranging from 360 to 770°C (Philips Electronics Instruments, Mahwah, NY, U.S.A.). The filament was placed into a 1.5 mm I.D. Pyrex glass tube, which was then inserted into the injection port. During this procedure, the backflush valve was open to prevent air from entering

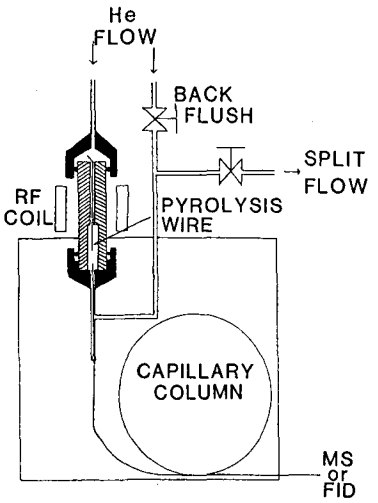

Fig. 1. Schematic diagram of the Curie-point Py–GC system.

the chromatographic system. The valve was closed after 30 s, and the pyrolyzer was fired for 10 s.

The separation was achieved on a 7.2 m × 0.2 mm I.D. capillary column, coated with 0.11 μm of DB5 (J&W Scientific, Folsom, CA, U.S.A.), using a HP 5890 gas chromatograph, equipped with a flame-ionization detector and HP 5970 series mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). The mass-selective detector was operated at 70 eV. Electron impact (EI) mass spectra were collected from 40 to 400 a.m.u. at a rate of 0.74 scans per s. The column temperature was programmed from 100 to 250°C at 8°C/min and at 20°C/min to 300°C, using a helium pressure of 90 kPa. The injector was kept at 290°C, and the split ratio was adjusted for optimum chromatographic performance.

RESULTS AND DISCUSSION

One of the requirements for the pyrolytic release of cellular fatty acids from whole microorganisms is the preservation of the structural integrity of the com-

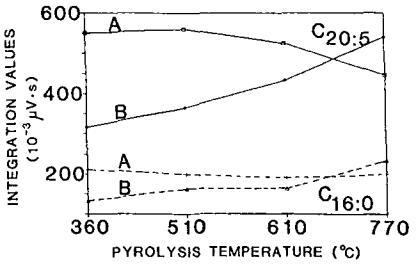

Fig. 2. Desorption efficiency of $C_{16:0}$ and $C_{20:5}$ fatty acid methyl esters at different pyrolysis temperatures. Curve A: power supply operated at full power (75 W), curve B: power supply operated at one-third power. Chromatographic conditions are described in the Experimental section.

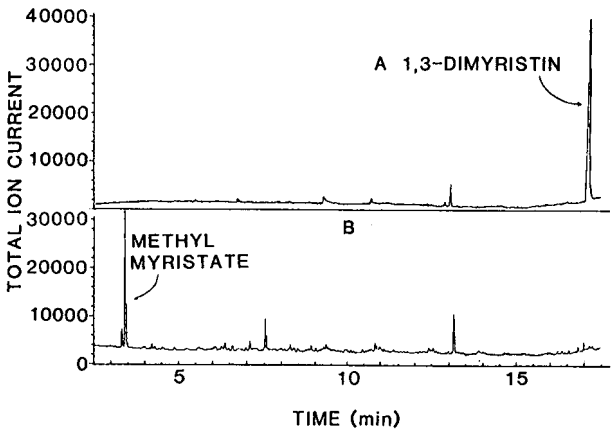

Fig. 3. Py–GC–MS of 1,3 dimyristin. (A) 1,3-dimyristin, (B) 1,3-dimyristin in presence of TMAH. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 610°C.

ponents. In addition, the method requires that the free as well as glyerol-bound fatty acids be liberated from the organisms as their corresponding methyl esters. Thus, desorption rather than thermal degradation of the fatty acid fraction is desired. These conditions could be established with the newly developed pyrolyzer, with its short heat-up rates, low dead volume, and fast removal of the released compounds on to the GC column.

First, the stability of several fatty acid methyl esters was examined under pyrolysis conditions. A mixture of methylpalmitate ($C_{16:0}$), methyleicosapentaeno-

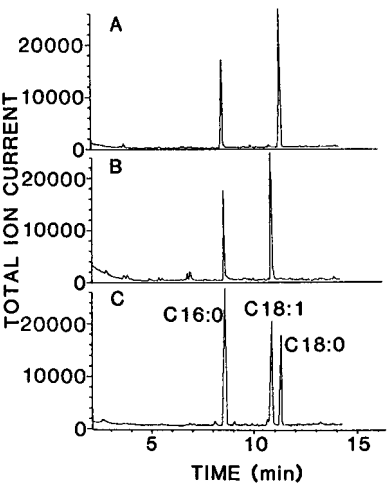

Fig. 4. Py–GC–MS of triglycerides and phosphoglycerides in presence of TMAH. (A) 1,2-distearoyl-3-palmitoyl glycerol–TMAH, (B) 1,3-dioleoyl-2-palmitoyl glycerol–TMAH, (C) phosphatidic acid–TMAH. The chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

ate($C_{20:5}$) and 2-hydroxymethylpalmitate was applied to a 610°C Curie-point wire in amounts ranging from 50 to 200 ng per component. The chromatographic analyses showed no evidence of thermal degradation. Recovery of the fatty acid methyl esters was quantitative, within the margin of error due to the application of the sample solution to the wire. Similar results were observed when the methyl esters of $C_{20:5}$ and $C_{16:0}$ were desorbed at different temperatures (Fig. 2). The higher final pyrolysis temperatures did not result in thermal degradation of the compounds examined. The effects of decreased power, which results in longer heat-up rates, was also examined with the same fatty acid methyl ester mixture. If operated at full power (75 W), the temperature rise time was less than 400 ms. Fig. 2 shows the desorption efficiency of the two fatty acid methyl esters at one-third power, which should result in a *ca.* three-fold decrease of the heating rate. The desorption efficiency at the highest pyrolysis temperature (770°C) was similar for the slow and fast heating rates; however, it dropped somewhat at lower pyrolysis temperatures when the heat-up rates were greater. The 75 W power supply produced consistent desorption efficiencies at different pyrolysis temperatures; however, for some applications a more powerful pyrolyzer may be desirable.

The reactions of TMAH with a number of saturated, unsaturated, and hydroxy fatty acids were investigated at different pyrolysis temperatures. Typically, 1 μl of a chloroform solution, containing the free fatty acids was transferred to the wire. After the solvent had evaporated, 10 μl of a 1% methanolic TMAH solution was applied. In all cases, the reaction with the corresponding methyl esters was complete. This was verified by the disappearance of the underivatized acids from the chromatogram. Hydroxy fatty acids were only methylated at the carboxylic function.

In a different set of experiments, the reaction between diacylglycerides, triacylglycerides, phosphoglycerides and TMAH was examined. The top tracing in Fig. 3 shows 1,3-dimyristin, analyzed by desorption from a 610°C Curie-point wire. The lower tracing was obtained after treating the wire with TMAH. The compound was identified by its mass spectrum as methylmyristate. The reaction was assumed to

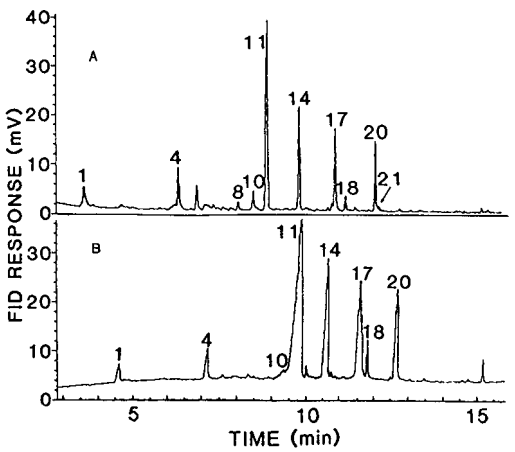

Fig 5. Py–GC–FID of *E. coli* cells. (A) *E. coli* cells in presence of TMAH, (B) *E. coli* cells. The names of the compounds are listed in Table I. The peaks in chromatogram B represent free fatty acids. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

TABLE I
PEAK IDENTIFICATION OF FATTY ACID METHYL ESTERS IN FIGS. 5–12

Peak No.	*Fatty acid methyl ester*	*Peak No.*	*Fatty acid methyl ester*
1	$C_{12:0}$	12	*iso*-$C_{17:0}$
2	2-OH-$C_{12:0}$	13	*anteiso*-$C_{17:0}$
3	3-OH-$C_{12:0}$	14	cyclopropyl-C_{17}
4	$C_{14:0}$	15	$C_{17:0}$
5	*iso*-$C_{15:0}$	16	*iso*-$C_{18:0}$
6	*anteiso*-$C_{15:0}$	17	$C_{18:1}$
7	$C_{15:0}$	18	$C_{18:0}$
8	3-OH-$C_{14:0}$	19	*iso*-$C_{19:0}$
9	*iso*-$C_{16:0}$	20	isopropyl-C_{19}
10	$C_{16:1}$	21	$C_{19:0}$
11	$C_{16:0}$	22	$C_{20:0}$

be quantitative, since the diacylglyceride was absent in the chromatogram. Similar experiments with distearoyl-palmitoyl glycerol, dioleoyl-palmitoyl glycerol and phosphatidic acid were carried out. The chromatograms in Fig. 4 show that the glycerides, pyrolyzed in the presence TMAH produced fatty acid methyl esters in ratios which corresponded to the actual distribution of these residues in the original molecules.

The flame ionization detection (FID) chromatograms in Figs. 5–11 are the fatty acid methyl ester profiles of a select group of microorganisms. The analyses were repeated using mass spectra detection, to confirm the identity of the fatty acid methyl esters. Fig. 5 shows the composition of the pyrolysis pattern of *E. coli* before and after the addition of TMAH. Previous experiments with acylglycerides showed no evidence

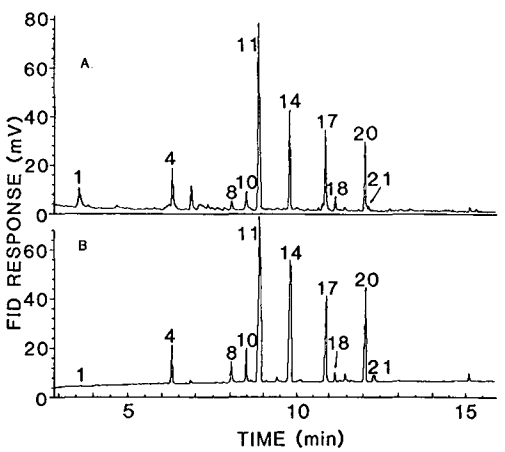

Fig. 6. Fatty acid methyl ester profiles of *E. coli* by Py–GC–FID. (A) *E. coli* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

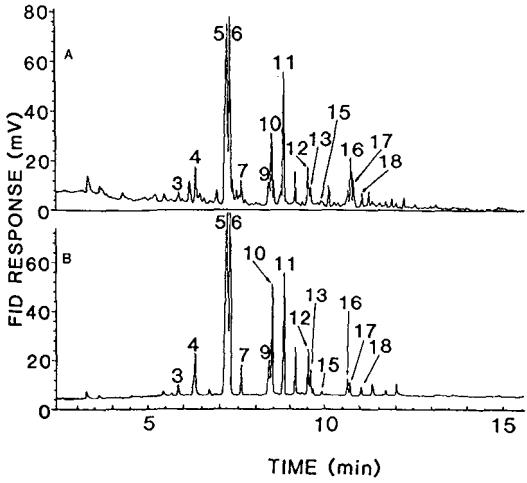

Fig. 7. Fatty acid methyl ester profiles of *P. maltophilia* by Py–GC–FID. (A) *P. maltophilia* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

of thermal degradation of these compounds to fatty acids. Thus, the free fatty acids, which are the major peaks in the bottom chromatogram, are actually part of the lipid content of the organism. The chromatogram above demonstrates the completeness of the reaction with TMAH to the corresponding methyl esters. The mixing of the reagent with the cells is important if consistent results are to be obtained. In a typical experiment, 1–5 mg of the cells are scraped from the agar plate. The cells were then suspended in 500 μl water and 5 μl of the suspension was transferred to the wire. During application, the wire was turned mechanically. Then, approximately 10 μl of

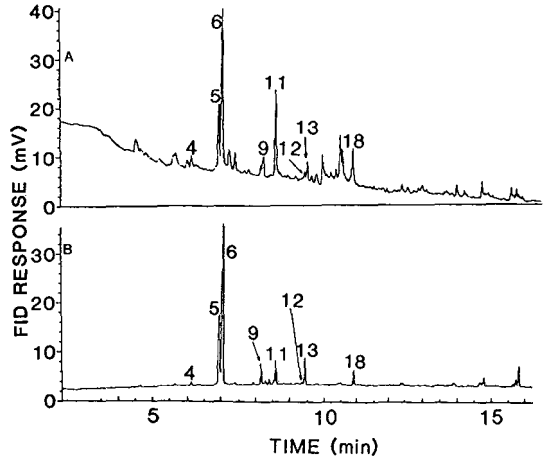

Fig. 8. Fatty acid methyl ester profiles of *M. luteus* by Py–GC–FID. (A) *M. luteus* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

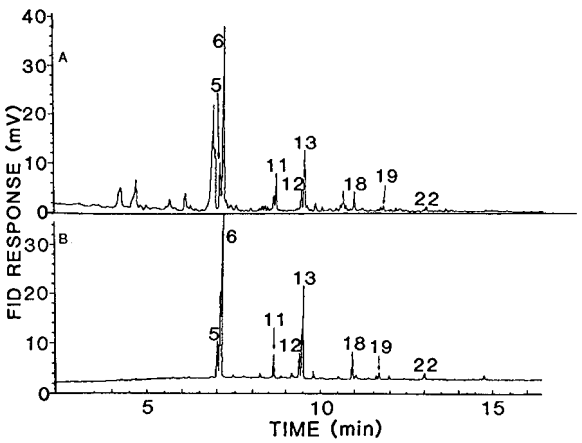

Fig. 9. Fatty acid methyl ester profiles of *S. aureus* by Py–GC–FID. (A) *S. aureus* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

the 1% methanolic TMAH solution was added to the turning wire and the aqueous methanol phase was removed by evaporation over a period of 5 min. This procedure deposited a uniform, thin film of cells on the wire and resulted in highly reproducible pyrograms. Mixing the reagent with water has no apparent effect on its reactivity with the complex lipids. The minimum number of cells necessary to obtain a well defined fatty acid methyl ester profile was determined from dry cell weights, as well as turbitity measurements. Pyrolysis of approximatey $5 \cdot 10^6$ cells produced a total ion chromatogram, which allowed mass spectral interpretation of components present at levels greater than 1% of the total amount of fatty acid methyl esters.

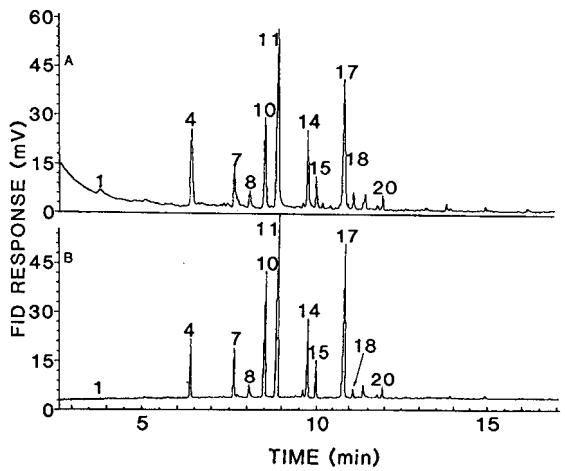

Fig. 10. Fatty acid methyl ester profiles of *E. aerogenes* by Py–GC–FID. (A) *E. aerogenes* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

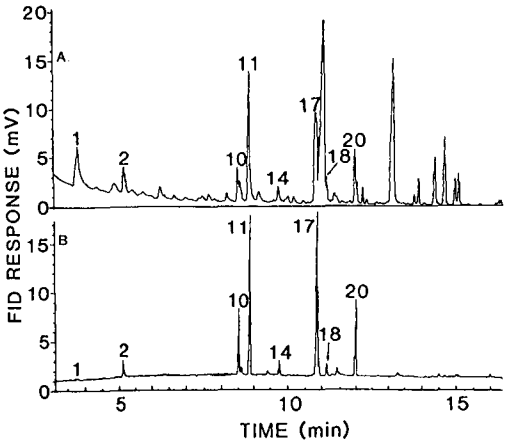

Fig. 11. Fatty acid methyl ester profiles of *P. aeruginosa* by Py–GC–FID. (A) *P. aeruginosa* cells/TMAH. (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

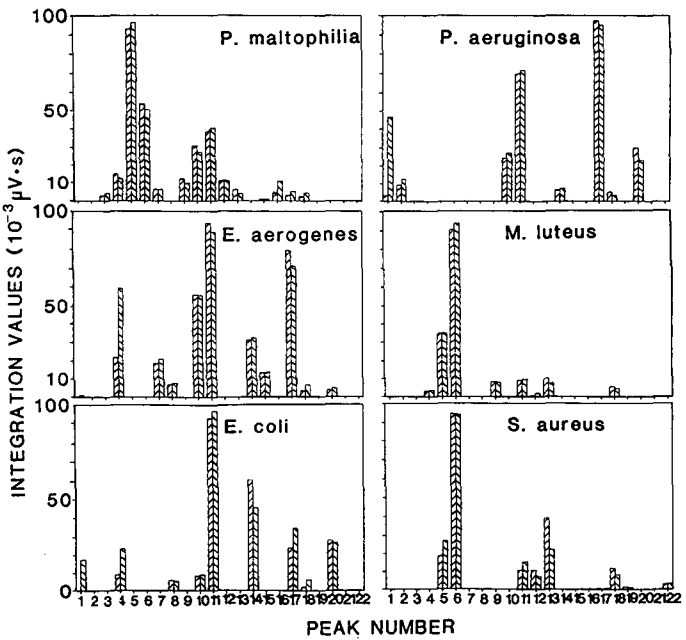

Fig. 12. Comparison of microbial fatty acid methyl ester profiles from whole cells and lipids extracts. Right-hand columns: whole cell pyrolysis in presence of TMAH; left-hand columns: fatty acid methyl ester extracts from the corresponding microorganisms. The original integration values have been multiplied by scaling factors for comparative representation. The fatty acids, identified by peak numbers, are listed in Table I.

Figs. 6–11 illustrate the potential of the method to generate characteristic fatty acid methyl ester profiles of microorganisms without prior extraction of the lipids. The top chromatogram in each figure was obtained by pyrolysis of whole microorganisms in the presence of TMAH. The lower profile represents the fatty acid methyl ester distribution of the hydrolyzed and extracted lipids of the same organism. Extraction and derivatization were performed as outlined in the Experimental section. The fatty acid methyl ester profiles obtained for all microorganisms by the two different methods appear very similar. Closer inspection shows that all of the fatty acids detected in the extract were also present in the pyrolyzates of the cells, including the cyclopropyl and hydroxy fatty acids. A peak-by-peak comparison of the fatty acid profiles from the whole-cell pyrolysis and from the lipid extract is shown in Fig. 12. The overall agreement between the two methods is very good, with the exception of the low-molecular-weight fatty acids, which are recovered at much higher yields during pyrolysis. These compounds may be lost during the sample preparation of the extracted lipids in a step that requires the complete evaporation of the solvent. The ratios of the remaining normal, branched and unsaturated fatty acids are in relatively good agreement.

Some variation between the two methods may be introduced by thermal degradation products which are eluted near a fatty acid methyl ester. Such peaks can be seen in the chromatograms of *P. maltophilia*, *P. aeruginosa* and *M. luteus*. Even though there is not much interference in these particular cases, mass spectral detection with single-ion displays, a longer column, and a stationary phase more suitable for this type of separation will most certainly improve the analysis.

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SELECTIVITY OF AN OCTADECYL-MODIFIED VINYL ALCOHOL COPOLYMER GEL FOR THE RETENTION OF POLAR COMPOUNDS

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SUMMARY

The enthalpies of phenols and aromatic acids were measured in order to characterize their behaviour on an octadecyl-bonded vinyl alcohol copolymer gel in reversed-phase liquid chromatography. These values were compared with the enthalpies measured on an octadecyl-bonded silica gel. The enthalpies of non-polar compounds, measured on an octadecyl-bonded silica gel, were higher than those obtained on the bonded vinyl alcohol copolymer gel. However, the enthalpies of phenols and aromatic acids were higher than those obtained on an octadecyl-bonded silica gel. This means that hydrogen bonding may be involved in the retention of phenols and aromatic acids on this vinyl alcohol copolymer gel.

INTRODUCTION

The retention of hydrophobic compounds on an octadecyl-bonded vinyl alcohol copolymer gel was investigated in relation to their Van der Waals volumes and a selectivity index representing the energy effect related to delocalization energy as used previously in reversed-phase liquid chromatography[1,2]. The selectivity index, R^* related to various intermolecular interactions is given by the equation

$$R = R_v + R^*$$

where R is the retention time of the analyte and R_v is the molecular size index, defined as the retention of a hypothetical *n*-alkane having the same Van der Waals volume as the analyte. The retention time of highly hydrophobic compounds was longer than their predicted retention times on both an octadecyl-bonded silica gel[3] and an octadecyl-bonded vinyl alcohol copolymer gel[4]. The difference between the experimental and predicted capacity ratio could be related to their enthalpies[3,4]. The value of the enthalpy effect of alkylbenzenes in which the alkyl chain was longer than six carbon atoms increased dramatically with increasing chain length, but the value of the selectivity index became fairly constant[3,4]. Direct adsorption of solutes on the surface of these packings could therefore be the predominant force in the retention of the larger sized alkylbenzenes on an octadecyl-bonded vinyl alcohol copolymer gel and on an octadecyl-bonded silica gel. Further, the enthalpy effect of polyaromatic hydro-

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TABLE I

PHYSICAL PARAMETERS AND THE LOGARITHM OF CAPACITY RATIOS (k') OF PHENOLS

Experimental conditions: column, BHST 602 C_{18} (10 cm × 6 mm I.D.); eluent, 50% aqueous acetonitrile containing 0.05 *M* phosphoric acid; flow-rate, 1 ml/min.

No.	*Compound*	*VWV*[a]	*Log k'*								*S*[b]	$-\Delta H$	$-\Delta H^c$
			Temperature [1/T (K^{-1} · 10^5)]										
			309.6	*314.5*	*319.5*	*324.7*	*330.0*	*335.6*	*341.3*	*347.2*			
1	Phenol	53.88	−0.322	−0.298	−0.271	−0.242	−0.219	−0.193	−0.162	−0.126	9.218	2.344	1.980
2	2-Methylphenol	65.03	−0.166	−0.139	−0.104	−0.089	−0.064	−0.050	−0.018	0.015	8.127	2.088	1.683
3	4-Methylphenol	65.03	−0.241	−0.210	−0.180	−0.164	−0.139	−0.116	−0.084	−0.050	9.816	2.217	1.910
4	2,3-Dimethylphenol	76.18	−0.052	−0.029	0.004	0.027	0.050	0.063	0.090	0.125	8.330	2.062	1.926
5	2,4-Dimethylphenol	76.18	−0.080	−0.050	−0.018	−0.005	0.016	0.036	0.062	0.085	8.662	1.922	1.872
6	2,5-Dimethylphenol	76.18	−0.085	−0.054	−0.027	−0.012	0.007	0.028	0.057	0.094	8.683	2.023	1.880
7	2,6-Dimethylphenol	76.18	−0.050	−0.024	0.002	0.020	0.038	0.055	0.080	0.109	8.456	1.836	1.941
8	3,4-Dimethylphenol	76.18	−0.158	−0.130	−0.103	−0.083	−0.061	−0.042	−0.010	0.018	9.925	2.067	1.906
9	3,5-Dimethylphenol	76.18	−0.149	−0.119	−0.090	−0.074	−0.054	−0.034	−0.007	0.023	9.833	1.979	1.889
10	2,3,5-Trimethylphenol	87.33	0.031	0.055	0.088	0.110	0.132	0.144	0.168	0.211	9.247	2.043	1.947
11	2,3,6-Trimethylphenol	87.33	0.054	0.082	0.112	0.127	0.146	0.159	0.185	0.208	9.027	1.775	1.940
12	2,4,6-Trimethylphenol	87.33	0.052	0.073	0.105	0.122	0.139	0.148	0.171	0.192	9.101	1.635	1.898
13	2,3,5,6-Tetramethylphenol	98.48	0.164	0.188	0.217	0.234	0.251	0.261	0.282	0.309	–	1.654	1.960
14	2-Ethylphenol	75.26	−0.050	−0.021	0.011	0.030	0.053	0.070	0.097	0.115	8.272	1.968	1.877
15	3-Ethylphenol	75.26	−0.138	−0.107	−0.071	−0.043	−0.016	0.010	0.046	0.086	9.246	2.641	1.838
16	4-Ethylphenol	75.26	−0.142	−0.113	−0.082	−0.063	−0.045	−0.027	0.003	0.032	9.314	2.008	1.886

17	2-Chlorophenol	63.03	−0.078	−0.048	−0.015	0.006	0.034	0.059	0.081	0.138	6.661	2.445	1.899
18	3-Chlorophenol	63.03	0.018	0.054	0.093	0.119	0.153	0.173	0.210	0.264	5.261	2.817	2.118
19	4-Chlorophenol	63.03	−0.015	0.021	0.053	0.085	0.113	0.145	0.177	0.222	5.700	2.783	2.085
20	2,3-Dichlorophenol	72.51	0.182	0.219	0.257	0.285	0.315	0.340	0.377	0.417	4.362	2.752	2.061
21	2,4-Dichlorophenol	72.51	0.253	0.286	0.327	0.356	0.385	0.411	0.450	0.488	3.555	2.784	2.029
22	2,5-Dichlorophenol	72.51	0.249	0.289	0.325	0.353	0.383	0.404	0.442	0.447	3.511	2.663	2.031
23	2,6-Dichlorophenol	72.51	0.143	0.171	0.201	0.224	0.246	0.266	0.298	0.326	5.207	2.163	2.032
24	3,4-Dichlorophenol	72.51	0.266	0.308	0.349	0.381	0.413	0.442	0.482	0.521	3.149	3.006	2.133
25	3,5-Dichlorophenol	72.51	0.403	0.446	0.489	0.523	0.555	0.581	0.619	0.656	2.091	2.981	2.209
26	2,3,4-Trichlorophenol	81.99	0.459	0.496	0.542	0.579	0.615	0.629	0.663	0.704	3.063	2.880	2.083
27	2,3,5-Trichlorophenol	81.99	0.568	0.611	0.655	0.703	0.730	0.762	0.810	0.852	1.897	3.376	2.374
28	2,3,6-Trichlorophenol	81.99	0.382	0.412	0.447	0.478	0.503	0.524	0.560	0.587	3.442	2.463	2.093
29	2,4,5-Trichlorophenol	81.99	0.531	0.571	0.623	0.667	0.696	0.731	0.771	0.814	2.199	3.376	2.157
30	3,4,5-Trichlorophenol	81.99	0.590	0.634	0.678	0.728	0.752	0.784	0.826	0.872	1.651	3.319	2.335
31	2,3,4,5-Tetrachlorophenol	91.47	0.776	0.824	0.871	0.923	0.951	0.980	1.024	1.067	1.438	3.434	2.470
32	2,3,5,6-Tetrachlorophenol	91.47	0.675	0.709	0.754	0.791	0.810	0.831	0.866	0.904	2.880	2.670	2.251
33	Pentachlorophenol	100.95	0.885	0.926	0.972	1.012	1.029	1.051	1.088	1.127	2.497	2.791	2.439
34	3-Bromophenol	66.48	0.087	0.125	0.163	0.189	0.224	0.250	0.286	0.317	4.374	2.747	2.039
35	4-Bromophenol	66.48	0.060	0.099	0.138	0.165	0.197	0.228	0.262	0.301	4.578	2.842	2.024
36	2,4-Dibromophenol	79.08	0.400	0.443	0.484	0.520	0.550	0.577	0.612	0.654	3.041	2.974	2.021

[a] Van der Waals volume (cm^3/mol) calculated by Bondi's method.
[b] Selectivity index (units), obtained on BHST 602 C_{18}.
[c] Enthalpy effect (kcal/mol), obtained on ERC-1000 (ODS-silica gel) from ref. 7 (eluent, 70% aqueous acetonitrile containing 0.05 *M* phosphoric acid).

TABLE II

PHYSICAL PARAMETERS AND THE LOGARITHM OF CAPACITY RATIOS (k') OF AROMATIC ACIDS

Experimental conditions: column, BHST 602 C_{18} (10 cm × 6 mm I.D.); eluent, 30% aqueous acetonitrile containing 0.05 *M* phosphoric acid; flow-rate, 1 ml/min.

No.	*Compound*	*VWV*[a]	*Log k'*								*S*[b]	$-\Delta H$	$-\Delta H^c$
			Temperature [1/T (K^{-1} · 10^5)]										
			309.6	*314.4*	*319.5*	*324.7*	*330.0*	*335.6*	*341.3*	*347.2*			
1	Benzoic acid	65.36	0.063	0.102	0.146	0.186	0.232	0.278	0.332	0.370	9.131	3.796	2.400
2	2-Methylbenzoic acid	76.51	0.246	0.290	0.334	0.376	0.419	0.475	0.510	0.564	9.278	3.847	2.647
3	3-Methylbenzoic acid	76.51	0.269	0.307	0.358	0.399	0.443	0.495	0.535	0.586	9.264	3.869	2.555
4	4-Methylbenzoic acid	76.51	0.252	0.290	0.343	0.382	0.436	0.492	0.523	0.578	9.278	4.004	2.540
5	2,4-Dimethylbenzoic acid	87.66	0.460	0.502	0.553	0.604	0.648	0.697	0.742	0.796	9.423	4.085	2.769
6	2,5-Dimethylbenzoic acid	87.66	0.446	0.489	0.544	0.586	0.638	0.680	0.727	0.776	9.440	4.014	2.830
7	2,6-Dimethylbenzoic acid	87.66	0.106	0.145	0.186	0.218	0.254	0.298	0.328	0.372	9.791	3.193	2.311
8	3,4-Dimethylbenzoic acid	87.66	0.425	0.467	0.521	0.567	0.621	0.661	0.708	0.763	9.457	4.101	2.696
9	3,5-Dimethylbenzoic acid	87.66	0.480	0.520	0.574	0.615	0.660	0.714	0.752	0.801	9.416	3.926	2.754
10	2,4,6-Trimethylbenzoic acid	98.81	0.324	0.353	0.410	0.446	0.495	0.530	0.565	0.608	9.919	3.502	2.518
11	4-Ethylbenzoic acid	86.74	0.468	0.507	0.560	0.605	0.661	0.707	0.749	0.800	9.396	4.087	2.771
12	2-Chlorobenzoic acid	74.84	0.186	0.230	0.274	0.316	0.368	0.415	0.455	0.504	9.294	3.880	2.652
13	3-Chlorobenzoic acid	74.84	0.505	0.550	0.604	0.655	0.707	0.763	0.801	0.867	8.963	4.379	2.915
14	4-Chlorobenzoic acid	74.84	0.509	0.554	0.613	0.663	0.722	0.773	0.827	0.881	8.961	4.571	2.934
15	2,4-Dichlorobenzoic acid	84.32	0.665	0.712	0.768	0.824	0.872	0.926	0.976	1.036	9.094	4.502	3.051
16	2,5-Dichlorobenzoic acid	84.32	0.616	0.665	0.727	0.771	0.834	0.878	0.931	0.992	9.161	4.541	3.068

17	2,6-Dichlorobenzoic acid	84.32	0.298	0.333	0.381	0.409	0.448	0.492	0.533	0.577	9.499	3.374	2.539
18	3,4-Dichlorobenzoic acid	84.32	0.880	0.936	1.003	1.055	1.123	1.178	1.240	1.299	9.059	5.116	3.332
19	3,5-Dichlorobenzoic acid	84.32	0.976	1.030	1.092	1.158	1.208	1.263	1.312	1.368	8.765	4.778	3.283
20	2-Bromobenzoic acid	77.96	0.266	0.307	0.361	0.403	0.457	0.503	0.552	0.602	9.307	4.115	2.805
21	3-Bromobenzoic acid	77.96	0.596	0.641	0.706	0.757	0.821	0.867	0.917	0.972	8.961	4.620	3.075
22	4-Bromobenzoic acid	77.96	0.607	0.656	0.720	0.772	0.832	0.887	0.917	1.007	8.946	4.732	3.105
23	Phenylacetic acid	79.55	−0.136	−0.098	−0.063	−0.028	0.015	0.059	0.086	0.134	9.777	3.265	2.462
24	2-Tolylacetic acid	86.74	0.116	0.157	0.200	0.237	0.287	0.330	0.363	0.408	9.858	3.562	2.758
25	3-Tolylacetic acid	86.74	0.258	0.297	0.347	0.390	0.446	0.491	0.530	0.575	9.909	3.924	2.994
26	2-Chlorophenylacetic acid	85.07	0.172	0.049	0.094	0.125	0.172	0.209	0.240	0.283	9.705	2.187	2.544
27	4-Chlorophenylacetic acid	85.07	0.062	0.096	0.140	0.176	0.222	0.260	0.293	0.338	9.558	3.369	2.592
28	4-Phenyl-*n*-butyric acid	96.05	0.229	0.274	0.310	0.351	0.391	0.444	0.475	0.522	9.941	3.548	2.835
29	L(+)-Mandelic acid	80.18	0.588	−0.545	−0.519	−0.507	−0.475	−0.436	−0.422	−0.398	10.23	2.254	1.562
30	*trans*-Cinnamic acid	82.32	0.251	0.299	0.352	0.396	0.448	0.510	0.551	0.612	9.471	4.376	2.884
31	4-Methylcinnamic acid	93.47	0.467	0.512	0.571	0.621	0.687	0.738	0.788	0.856	9.592	4.738	3.039
32	3-Phenyl-*n*-propionic acid	86.04	0.060	0.102	0.145	0.182	0.220	0.275	0.306	0.355	9.789	3.563	2.767
33	Indole-3-acetic acid	91.65	−0.013	0.036	0.098	0.145	0.206	0.266	0.316	0.376	9.979	4.755	3.170
34	Indole-3-propionic acid	114.11	0.178	0.235	0.298	0.351	0.412	0.489	0.532	0.604	10.56	5.174	3.528
35	Indole-3-butyric acid	121.34	0.307	0.364	0.426	0.482	0.558	0.621	0.671	0.744	10.61	5.330	3.711
36	Hippuric acid	96.15	−0.729	−0.689	−0.671	−0.655	−0.633	−0.581	−0.586	−0.559	10.86	2.022	1.406

[a,b] See Table I.

[c] Enthalpy effect (kcal/mol), obtained on ERC-1000 (ODS-silica gel), from ref 12 (eluent, 30% aqueous acetonitrile containing 0.05 *M* phosphoric acid).

carbons was larger than that of alkylbenzenes on this vinyl alcohol copolymer gel compared with their retention on an octadecyl-bonded silica gel, which means that polyaromatic hydrocarbons are selectively retained on this organic packing[4].

The effect of temperature on the retention of phenols and aromatic acids was therefore measured, and their enthalpies were obtained in order to study the retention mechanism of polar compounds on this modified vinyl alcohol copolymer gel.

EXPERIMENTAL

The details of the chromatograph were described previously[4]. The 9-μm vinyl alcohol copolymer gel was BHST 602 C_{18} from Asahi Chemical (Kawasaki, Japan). The 10 cm × 6.0 mm I.D. packed column was thermostated at 15–50°C in a circulating water-bath (Ikedarika, Tokyo, Japan). The chemicals and their physical parameters are given in Table I.

RESULTS AND DISCUSSION

The capacity ratios of phenols, measured on an octadecyl-bonded vinyl alcohol copolymer gel at different temperatures, are given in Table I, together with their Van der Waals volumes, and the values for aromatic acids are given in Table II. The selectivity index was obtained from refs. 5 and 6. The enthalpy effect, measured on an octadecyl-bonded silica gel, was obtained from ref. 7. The correlation coefficient of the log k' values of phenols between this octadecyl-bonded vinyl alcohol copolymer gel and silica gel[2] was 0.889 ($n = 34$). The chromatographic behaviour of phenols on this organic polymer gel appeared to be similar to that on an octadecyl-bonded silica gel.

The correlation coefficient of the log k' values of aromatic acids between the octadecyl-bonded vinyl alcohol copolymer gel and the octadecyl-bonded silica gel[8] was 0.908 ($n = 35$), a value similar to that obtained for phenols.

The partition coefficient between octanol and water (log P) is a useful parameter for the optimization of reversed-phase liquid chromatography on polystyrene gel[9], and the correlation coefficient between the log P and log k' values, measured at 30°C, of phenols and aromatic acids was therefore calculated. The value was 0.915 ($n = 36$) and 0.858 ($n = 36$) for phenols and aromatic acids, respectively. These values are poor compared with those obtained on octadecyl-bonded silica gels[10,11], which indicates the existence of selectivity of this octadecyl-bonded vinyl alcohol copolymer gel in contrast to octadecyl-bonded silica gels. A further study was carried out of the difference in the enthalpy effects of phenols and aromatic acids.

The relationship between log k' for phenols and the reciprocal of the absolute temperature was linear. Although this linear relationship changed slightly at about 33°C, as found in the retention of alkyl compounds[4], no negative enthalpy effect was observed in the retention of phenols. The calculated values of the enthalpy effect for phenols are given in Table I. The enthalpy effect of alkylphenols was about 2.0 kcal/mol and the energy effect was about 9.0 units[5]. The enthalpy effect of halogenated phenols was about 2.9 kcal/mol. However, the energy effect decreased from 9.2 units for phenol to 2.5 units for pentachorophenol. Permethylphenols have lower enthalpy and perchlorophenols higher enthalpy than phenol, as shown in Figs. 1 and 2.

The enthalpy effect of alkylphenols was almost constant on octadecyl-bonded

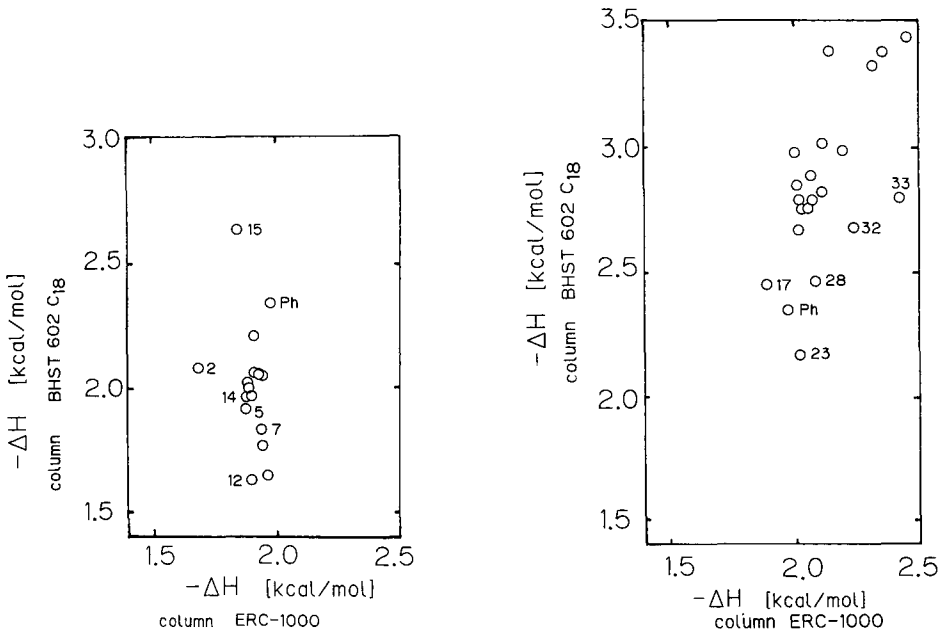
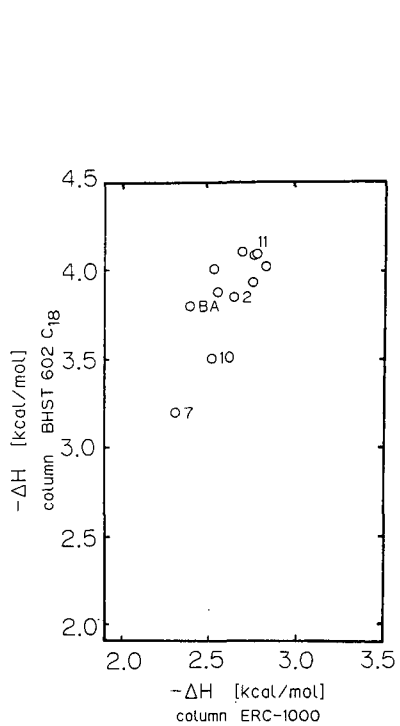


Fig. 1. Comparison of octadecyl-bonded vinyl alcohol copolymer and silica gels as a function of enthalpy for the retention of alkylphenols. For experimental conditions see Table I. Numbers next to symbols correspond to those in Table I. Ph = Phenol.

Fig. 2. Comparison of octadecyl-bonded vinyl alcohol copolymer and silica gels as a function of enthalpy for the retention of halogenated phenols. For experimental conditions, see Table I. Numbers next to symbols correspond to those in Table I.

silica gel, but on the organic polymer gel it varied from about 1.6 to 2.6 kcal/mol, as shown in Fig. 1. There was, therefore, no good correlation between the enthalpy of alkylphenols measured on the organic polymer gel and that measured on the silica gel ($r = 0.226, n = 16$). The enthalpy of halogenated phenols varied from about 1.9 to 2.5 kcal/mol on the octadecyl-bonded silica gel but from about 2.2 to 3.4 kcal/mol on the organic polymer gel. The correlation coefficient between the enthalpy measured on the organic polymer gel and that measured on the silica gel for halogenated phenols was 0.656 ($n = 21$). However, it was improved to 0.872 ($n = 17$) by excluding the *ortho*-substituted halogenated phenols. The correlation coefficient for phenols was 0.765 ($n = 36$). This poor correlation and the variation of the enthalpies of polar compounds indicate the existence of steric selectivity of this organic polymer gel. Their capacity ratios measured on the organic polymer gel were smaller than those measured on the octadecyl-bonded silica gel, whereas the enthalpy effect measured on the octadecyl-bonded vinyl alcohol copolymer gel was greater than that on the octadecyl-bonded silica gel. However, the enthalpies of hydrophobic compounds measured on the octadecyl-bonded silica gel were higher than those obtained on the organic polymer gel. This result indicates that phenols could further be adsorbed by hydrogen bonding on the organic polymer gel, even though the enthalpy effect was weaker than the selectivity index, and such adsorption was not the predominant retention force.



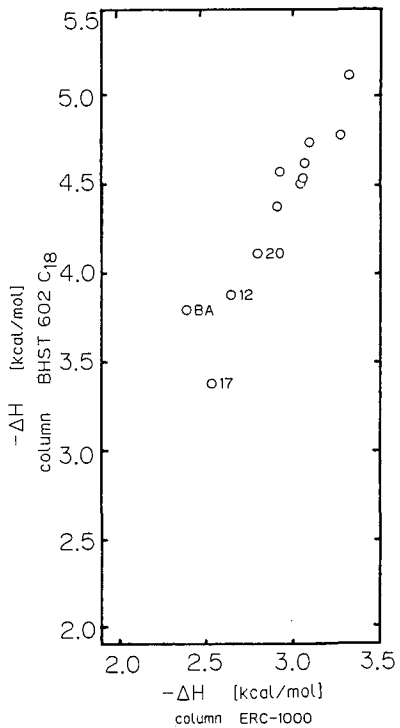


Fig 3. Comparison of octadecyl-bonded vinyl alcohol copolymer and silica gels as a function of enthalpy for the retention of alkylbenzoic acids. For experimental conditions, see Table II. Numbers next to symbols correspond to those in Table II. BA = Benzoic acid.

Fig. 4. Comparison of octadecyl-bonded vinyl alcohol copolymer and silica gels as a function of enthalpy for the retention of halogenated benzoic acids. For experimental conditions, see Table II. Numbers next to symbols correspond to those in Table II.

A similar result was obtained for the chromatographic behaviour of aromatic acids, their capacity ratios being small and their enthalpy effect large on the organic polymer gel. The enthalpy effect varied from about 3.2 to 5.1 kcal/mol on the octadecyl-bonded vinyl alcohol copolymer gel and from about 2.3 to 3.3 kcal/mol on the octadecyl-bonded silica gel[12], as shown in Figs. 3 and 4.

The correlation coefficient between the enthalpy of aromatic acids (n = 36) measured on the organic polymer gel and that on the silica gel was 0.899; the value was 0.942 (n = 22) only for benzoic acids. The enthalpy effect of aromatic acids was stronger than that of phenols on the octadecyl-bonded vinyl alcohol copolymer gel. Further, *ortho*-disubstituted aromatic acids were less retained on the organic polymer gel, as with substituted phenols. These results indicate that the carboxyl group of benzoic acids is involved in their retention on the octadecyl-bonded vinyl alcohol copolymer gel, even though their enthalpy effect was weaker than their selectivity index.

This selective retention of polar compounds on the organic polymer gel, compared with their chromatographic behaviour on octadecyl-bonded silica gel, may

be the reason why the vinyl alcohol copolymer gel was a powerful packing for the direct analysis of target compounds in urine and serum[13].

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CHARACTERISATION OF PRODUCTS FORMED BY CONDENSATIONS OF PRIMARY AMINES WITH POLYGODIAL, AND THEIR SCOPE FOR ENANTIOMER ANALYSES

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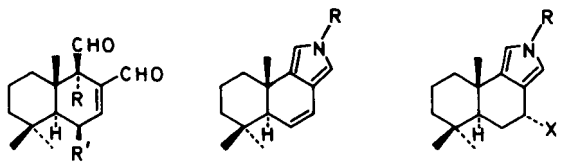
SUMMARY

The sesquiterpenedial polygodial undergoes smooth reactions with primary amines, under mild conditions, to give stable derivatives (1,3,4-trisubstituted pyrroles) useful for characterisation by gas chromatography–mass spectrometry. Diastereomeric pyrroles formed from chiral amines were in suitable instances separable by gas chromatography. With hydroxy amines it may be advantageous to convert the initial products into O-trimethylsilyl or O-*tert.*-butyldimethylsilyl ethers for analysis. All the derivatives studied yielded molecular ions under electron impact, while informative fragmentations also occurred.

INTRODUCTION

(−)-Polygodial (**1a**)[1] is the simplest member of a group of natural drimenedials, many of which show interesting biological activities. A convenient method for the analysis of enantiomeric polygodials is based on their rapid reaction with chiral amines to give stable diastereomeric pyrrole derivatives (**2**), separable by gas chromatography (GC)[2]. It is possible to employ a similar process for the analysis of chiral primary amines[3]. This report is concerned with qualitative work on a wide range of amines,



1a: R,R'=H
b: R=OH,R'=OOCCH$_3$

2

3

including biogenic amines and hydroxy amines. Derivatives containing hydroxyl groups were generally examined as trimethylsilyl (TMS) or *tert.*-butyldimethylsilyl (TBDMS) ethers. Of the fifteen amines included in this report, ten afforded derivatives that were either partially or fully resolved by capillary GC. Mass spectra of the pyrroles

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were structurally informative, and in many cases yielded intense ions, suitable for selective detection of primary amines.

EXPERIMENTAL

Solvents and reagents

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *tert.*-butyldimethylchlorosilane–imidazole (TBDMCS–imidazole) were obtained from Pierce and Warriner (Chester, U.K.). Ethyl acetate (Nanograde) was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), and pyridine (AnalaR grade, BDH, Poole, U.K.) was dried over potassium hydroxide pellets and redistilled prior to use.

Amines and other reference compounds

The primary amines were numbered as in Fig. 1. Compound **4** was a gift from Smith Kline and French. Compound **5** was obtained from Norse Labs. (Santa

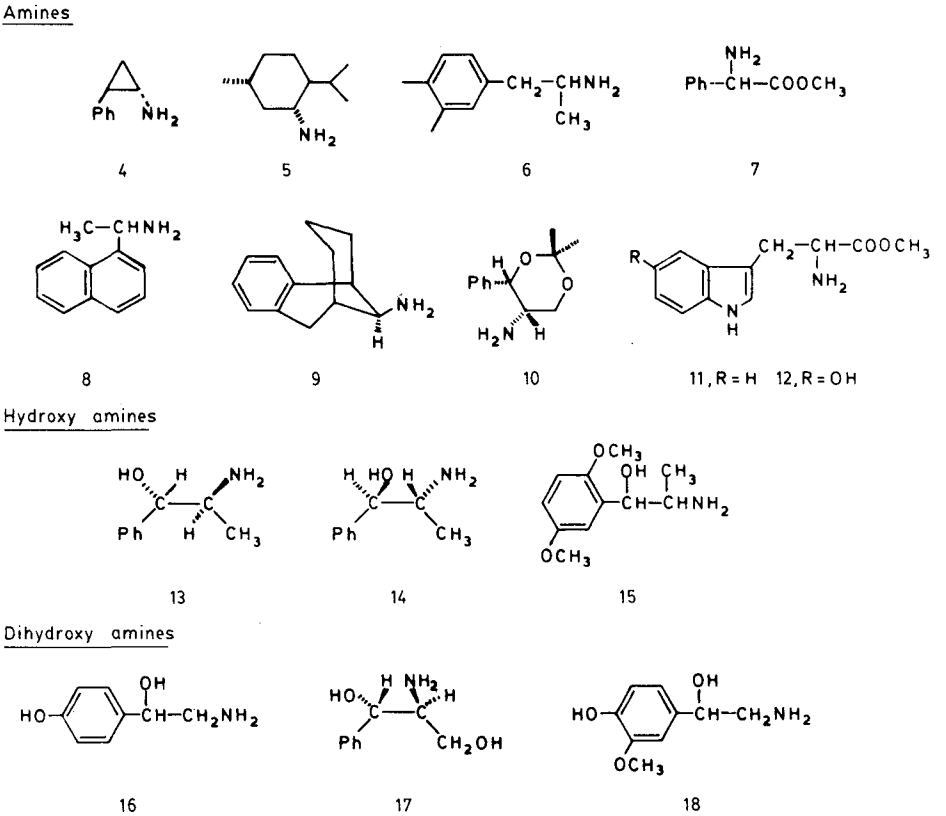

Fig. 1. Structures of amines. **4** = (±)-tranylcypromine; **5** = (−)-menthylamine; **6** = xylopropamine; **7** = D-(−)-phenylglycine methyl ester; **8** = (+)- and (−)-α-1-naphthylethylamine; **9** = Organon compound 6328 [5,6,7,8,9,10-hexahydro-5,9-methanobenzocycloocten-11-amine (5α,9α,11β)]; **10** = 4*S*,5*S*-(+)-5-amino-2,2-dimethyl-4-phenyl-1,3-dioxan; **11** = (±)- and L-(−)-tryptophan methyl ester; **12** = (±)-5-hydroxytryptophan methyl ester; **13** = D-(+)-norpseudoephedrine; **14** = L-(+)-norephedrine; **15** = (±)-methoxamine; **16** = (±)-octopamine; **17** = 1*S*,2*S*-(+)-2-amino-1-phenylpropane-1,3-diol; **18** = (±)-normetanephrine. Ph = Phenyl.

Barbara, CA, U.S.A.) and **6** was a gift from Roche (Welwyn Garden City U.K.). Compound **7** was purchased from Fluka (Fluorochem, Glossop, U.K.) and **8** (+) and (−) and **9** (+) were gifts from Organon Laboratories (Newhouse, U.K.). Compounds **10** and **11** were obtained from Sigma (Poole, U.K.) and **12** was a gift from Dr. E. C. Horning. Compounds **13** and **14** were purchased from Koch Light (A. and J. Beveridge, Edinburgh, U.K.) and **15** was a gift from The Wellcome Foundation (Dartford, U.K.). Compounds **16** and **18** were obtained from Calbiochem (San Diego, CA, U.S.A.) and **17** from Aldrich (Gillingham, Dorset, U.K.). Compounds **7** and **12**, originally obtained as free acids, were methylated prior to use. Dr. J. A. Pickett (Rothamsted) provided gifts of (±)- and (−)-polygodial.

Gas chromatography

Open-tubular GC was performed with a Hewlett-Packard (Winnersh, U.K.) 5880A gas chromatograph, equipped with CP-Sil 5 CB (Chrompack, London, U.K.) and SE-54 (GC^2, Northwich, Chester, U.K.) fused-silica capillary columns (25 m × 0.32 mm I.D.). The Grob-type injectors were operated in split mode (50:1) and the helium carrier gas flow-rates were 3 ml/min. The columns were operated according to conditions in Table I, while the instrument employed flame ionisation detectors.

Gas chromatography–mass spectrometry

GC–mass spectrometry (MS) was carried out with an LKB 9000 instrument, equipped with a DB-1 fused-silica capillary column, 60 m × 0.32 mm I.D. (J. and W. Scientific, Rancho Cordova, CA, U.S.A.) and a falling-needle injection system[4]. Helium carrier- and make-up gas flow-rates were 7 ml/min (measured at atmospheric pressure) and 25 ml/min, respectively. Mass spectra (22 eV) were recorded under electron-impact conditions: filament current, 4 A; trap current, 60 μA; accelerating voltage, 3.5 kV; and source and separator temperatures, 270°C.

Preparation of amine–polygodial condensation products

Primary amines (*ca.* 200 μg) were dissolved in water (50 μl) and mixed with 50 μl of a 1-μg/μl solution of (±)- or (−)-polygodial in ethyl acetate and with pyridine (10 μl). The mixture was shaken periodically for 2 h at room temperature. The aqueous phase was removed with a syringe, and the organic phase was washed with water (50 μl) and dried over anhydrous sodium sulphate. The solution was filtered through a small plug of cotton wool and analysed by capillary GC and GC–MS. The 1,3,4-trisubstituted pyrrole obtained from methoxamine (**15**) was treated with an additional quantity of (±)-polygodial (50 μg) and extracted and analysed as above.

In a study to determine possible competitive reactions of secondary amines, additional condensation reactions were performed, using (−)-amphetamine (50 μg)/polygodial (50 μg) and (−)-amphetamine (50 μg) + N-methylamphetamine (50 μg)/(±)polygodial (100 μg). The two solutions were allowed to react, and the mixtures were extracted and analysed as above.

Trimethylsilylation. Compounds **12–18** were converted into their corresponding 1,3,4-trisubstituted pyrrole products as above. After filtration and evaporation to dryness, the products were treated with BSTFA (20 μl) and dry pyridine (10 μl) and heated at 80°C for 30 min. The reagents were removed by evaporation, and the mono- or di-TMS ethers of the substituted pyrroles were redissolved in ethyl acetate for GC and GC–MS analyses.

tert.-Butyldimethylsilylation. The 1,3,4-trisubstituted pyrrole products of compounds **13** and **17** were prepared as above. Following filtration and evaporation to dryness, the compounds were treated with TBDMCS–imidazole (20 μl) and dry pyridine (10 μl) and heated at 80°C for 30 min. After removal of solvent, the corresponding pyrrole mono- and di-TBDMS ethers were extracted into ethyl acetate and analysed by GC and GC–MS.

RESULTS AND DISCUSSION

General features of the reactions

The reaction of polygodial with methylamine in aqueous buffer has been studied in some detail, and is known to lead to compounds of type **3** [X = OH (refs. 5–7) or $NHCH_3$ (ref. 8)]: these readily undergo thermal elimination during GC to afford the unsaturated products (**2**). In the present work, the aim was to determine the extent to which diastereomers (**2**) from various types of amines (Fig. 1) would be separable by GC. Optimisation of yields was not attempted, but in general the reactions proceeded smoothly, and on the analytical scale there was little or no evidence of by-products. The products from valine methyl ester (not included in this paper) and tranylcypromine (Fig. 1, No. **4**) were shown to be stable during two years' storage in the dry state. Most of the products were stored for several weeks as solutions in ethyl acetate without undergoing any significant changes, as judged by GC–MS.

Possible interference by secondary amines has not been explored in detail, but the reaction of amphetamine with polygodial[2] yielded identical results (by GC) in the absence or the presence of N-methylamphetamine.

GC properties

Retention indices (*I*) of the condensation products, analysed on the principal column used (CP-Sil 5 CB) ranged from 2466, for the unresolved menthylamine derivatives, to 3515 and 3521 for those of 5-hydroxytryptophan methyl ester as trimethylsilyl ethers. Data are summarised in Table I.

(i) Amines lacking other functional groups. Seven amines of this type (Fig. 1, **4**–**10**) were studied, of which only two yielded diastereomeric pyrroles that were separable by GC under the conditions used. The racemic adrenergic compound xylopropamine (**6**) afforded well-resolved products (ΔI = 9: Fig. 2). An equally satisfactory separation (ΔI = 11: Fig. 3) was observed for the pyrroles formed from (±)-polygodial and compound **10** (the acetonide of **17**): the diastereomer from (−)-polygodial was eluted first on the CP-Sil 5 CB column.

(ii) Tryptophan and 5-hydroxytryptophan methyl esters. A partial separation (ΔI = 6) of diastereomeric products from (±)-tryptophan methyl ester (**11**) was observed (Fig. 4, A). As indicated in Table I, the (+)-tryptophan methyl ester–(−)-polygodial product was eluted first.

Racemic 5-hydroxytryptophan methyl ester (**12**) yielded diastereomeric pyrroles, which, after trimethylsilylation, gave partially separated peaks (ΔI = 7; Fig. 4, B). The order of elution was not established, but probably corresponded to that of the tryptophan methyl ester derivatives.

(iii) β-Hydroxy amines. Three representative bioactive β-hydroxy amines each gave pyrroles that were sufficiently separated by GC for characterisation of the

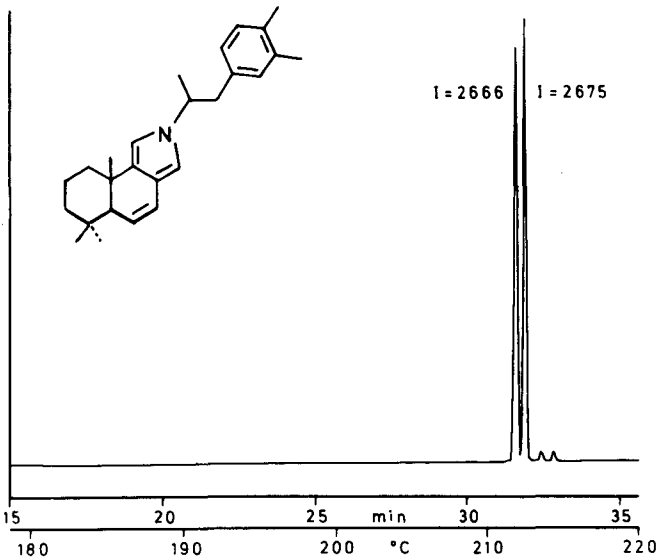

Fig. 2. GC separation of reaction products of xylopropamine with (±)-polygodial. Column, CP-Sil 5 CB fused-silica capillary (25 m × 0.32 mm I.D.); column temperature, programmed from 80°C (2 min) to 160°C (1 min) at 30°C/min, and then at 2°C/min to 270°C; helium flow-rate, 3 ml/min.

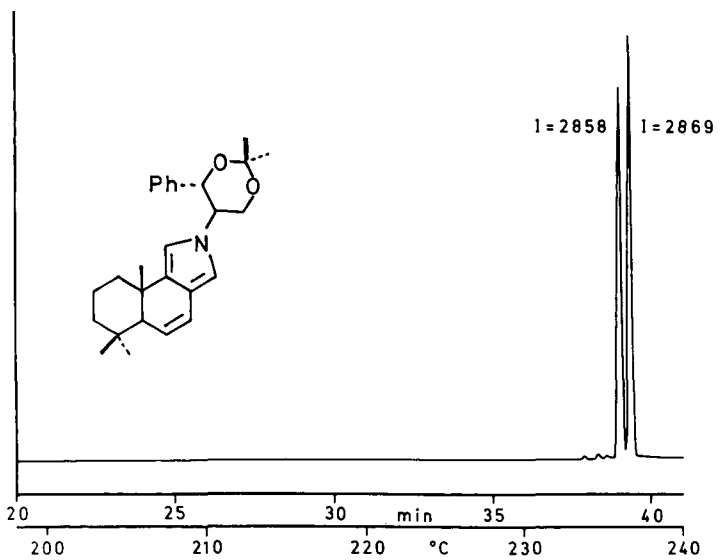

Fig. 3. GC separation of reaction products of 4*S*,5*S*-(+)-5-amino-2,2-dimethyl-4-phenyl-1,3-dioxan with (±)-polygodial. Column, SE-54 fused-silica capillary (25 m × 0.32 mm I.D.); column temperature, programmed from 80°C (2 min) to 170°C (1 min) at 30°C/min, and then at 2°C/min to 270°C; helium flow-rate, 3 ml/min. Ph = phenyl.

TABLE I

KOVÁTS RETENTION INDICES (*I*) AND MASS SPECTROMETRIC DATA (22 eV) FOR POLYGODIAL (PG) PRIMARY AMINE CONDENSATION PRODUCTS

Compound	*PG type*	*I*		$M^{+\cdot}$	*Base Peak*[c]	*m/z for other principal ions (intensities relative to base peak in parentheses)*[d]							
		CP-Sil 5	*SE-54*										
4	(±)	2713[a]	–	331(100)	331	333(5)	332(31)	330(12)	318(3)	317(16)	316(69)(**a**)	262(4)	261(3)
						260(4)	255(12)	254(47)	250(5)	249(21)(**g**)	248(24)(**h**)	247(4)	246(5)
						195(3)	170(6)(**l**)	118(4)	117(33)				
5	(±)	2466[a]	–	353(84)	338(**a**)	355(4)	354(26)	352(8)	340(5)	339(29)	284(6)	282(3)	272(15)
						271(73)(**g**)	270(43)(**h**)	269(5)	268(5)	242(4)(**b**)	229(6)	215(3)	214(3)
						201(4)	200(22)(**j**)	144(3)	132(10)(**k**)	83(5)			
6	(±)	2666[a]	–	361(54)	242(**b**)	363(3)	362(20)	360(6)	359(3)	348(3)	347(12)	346(38)(**a**)	345(4)
		2675				292(3)	290(3)	281(4)	280(6)	279(26)(**g**)	278(18)(**h**)	277(4)	244(7)
						243(12)	200(4)(**j**)	160(8)	158(4)(**f**)	148(3)	147(13)	146(8)	144(6)
						132(4)(**k**)	131(5)	120(4)	119(8)(**d**)				
7	(–)	2656[a]	2705[a]	363(80)	348(**a**)	365(6)	364(28)	362(8)	350(7)	349(27)	306(3)	305(4)	304(10)
						294(7)	293(5)	292(10)	283(3)	282(18)	281(76)(**g**)	280(62)(**h**)	279(12)
						278(4)	220(3)	214(4)	150(4)	149(24)(**d**)	145(3)	144(5)	132(5)(**k**)
						122(5)	121(32)	91(4)					
8(–)	(–)	2977[b]	3043[b]	369(45)	155(**d**)	370(14)	368(3)	355(4)	354(18)(**a**)	288(8)	287(27)(**g**)	286(14)(**h**)	215(5)
8(+)	(–)	2977[b]	3043[b]			214(5)	201(4)	200(24)(**j**)	156(18)	154(8)	153(4)	152(3)	145(4)
						144(7)	141(3)	133(8)	132(10)(**k**)	131(5)			
9	(±)	3198[b]	–	385(97)	370(**a**)	387(7)	386(32)	384(10)	383(4)	373(3)	372(11)	371(38)	316(5)
						315(3)	314(4)	305(6)	304(29)	303(94)(**g**)	302(50)(**h**)	301(11)	300(4)
						216(5)	201(4)	200(22)(**j**)	172(8)	171(27)	170(7)(**l**)	158(3)(**f**)	146(3)
						144(6)	143(8)	142(5)	132(8)(**k**)	130(6)	129(20)	117(4)	115(4)
10	(±)	2798[b]	2858[b]	405(100)	405	407(8)	406(26)	404(8)	403(3)	392(5)	391(23)	390(63)(**a**)	389(5)
		2809	2869			324(9)	323(39)(**g**)	322(26)(**h**)	321(4)	320(3)	317(5)	302(5)	298(3)
	(–)	2797[b]				292(3)	243(3)	242(11)(**b**)	241(56)	240(5)	234(4)	233(4)	228(7)
						227(13)	226(70)(**c**)	198(4)	184(3)	172(8)	171(6)	170(12)(**l**)	160(10)
						159(67)	158(35)(**f**)	157(9)	156(3)	144(3)	133(4)	130(3)	106(3)
						105(5)	77(3)						
11(±)	(±)	3332[b]	3416[b]	416(52)	130(**d**)	418(6)	417(18)	415(8)	414(6)	403(3)	402(10)	401(29)(**a**)	400(6)
		3339	3423			357(3)	355(2)	335(5)	334(20)(**g**)	333(12)(**h**)	332(3)	287(9)	286(8)
11(–)	(–)	3338[b]				272(4)	216(3)	215(3)	205(4)	204(5)	203(3)	202(7)	201(5)
						200(7)(**j**)	160(3)	144(3)	132(4)(**k**)	131(12)	129(5)		
12 di-TMS	(±)	3515[b]	3570[b]	576(6)	290(**d**)	577(3)	561(3)(**a**)	292(9)	291(26)				
		3521	3576										

13	(±)	2673[a]		349(4)	158(**f**)	347(5)	345(4)	332(15)	331(28)	330(7)	317(8)	316(22)	274(3)
		2678				265(3)	263(3)	262(3)	260(3)	250(5)	249(14)	248(14)	247(4)
	(−)	2672[a]				246(6)	243(7)	242(68)(**b**)	241(92)	240(8)	228(4)	227(21)	226(94)(**c**)
						225(6)	224(3)	215(5)	213(3)	200(8)(**j**)	198(7)	185(5)	184(9)
						183(4)	173(4)	172(12)	171(9)	170(43)(**l**)	168(3)	160(8)	159(70)
						157(28)	156(20)	144(10)	143(7)	133(6)	132(5)(**k**)	131(4)	130(4)
						129(4)	118(5)	117(7)	107(10)(**d**)	106(64)	105(72)	78(18)	77(50)
13 TMS	(±)	2598[a]		421(6)	242(**b**)	422(3)	244(3)	243(18)	179(6)(**d**)				
13 mono-	(±)	2839[a]		463(8)	242(**b**)	464(3)	406(3)	243(16)	221(4)(**d**)				
-TBDMS		2846											
14	(±)	2641[a]		349(13)	242(**b**)	350(5)	348(5)	347(6)	334(4)(**a**)	333(3)	332(6)	331(10)	318(5)
		2650				316(9)	249(5)	244(7)	243(16)	241(50)	240(4)	228(6)	227(8)
						226(56)(**c**)	184(3)	183(4)	172(8)	171(4)	170(11)(**l**)	160(4)	159(28)
						158(26)(**f**)	157(8)	156(6)	107(3)	106(35)	105(27)	77(13)	
14 TMS	(±)	2599[a]		421(15)	242(**b**)	422(5)	244(5)	243(22)	179(3)(**d**)				
		2611											
	(−)	2598[a]											
15	(±)	2982[b]		409(9)	226(**c**)	407(3)	405(3)	393(5)	392(14)	391(40)	390(6)	378(4)	377(12)
		3004				376(39)	362(9)	361(22)	360(80)	359(4)	344(4)	310(4)	309(14)
						308(20)	278(6)	276(7)	275(4)	244(4)	243(15)	242(64)(**b**)	241(92)
						240(9)	229(3)	228(6)	227(21)	215(6)	200(5)(**j**)	198(4)	185(4)
						184(10)	183(5)	173(4)	172(14)	171(11)	170(35)(**l**)	167(10)	166(76)(**m**)
						165(10)	160(6)	159(50)	158(65)(**f**)	157(21)	156(15)	155(4)	151(20)
						150(4)	149(8)	148(9)	144(9)	143(5)	137(7)	136(6)	135(6)
						134(6)	133(6)	132(7)(**k**)	131(6)	123(15)	121(6)	120(15)	109(6)
						108(6)	107(4)	106(11)	95(12)	93(4)	92(4)	80(4)	77(4)
15 TMS	(±)	2886[b]		481(19)	242(**b**)	483(4)	482(8)	466(4)	244(4)	243(20)	241(7)	240(16)	239(77)(**d**)
		2910				238(3)	226(3)(**c**)	158(3)(**f**)	77(5)				
16 di-TMS	(−)	2909[a]	2943[a]	495(9)	267(**d**)	496(3)	480(3)	405(5)	390(4)	269(12)	268(23)		
		2916	2950										
17 di-TMS	(±)	2721[b]	2746[b]	509(18)	330(**e**)	511(3)	510(9)	495(2)	494(4)(**a**)	332(8)	331(29)	179(6)(**d**)	
17 mono-	(±)		3127[b]	479(36)	372(**e**)	481(5)	480(14)	465(7)	464(17)(**a**)	422(4)	397(4)(**g**)	396(5)(**h**)	374(10)
-TBDMS			3132			373(33)	371(4)	356(2)	315(3)	290(4)	288(3)	242(3)(**b**)	241(12)
						226(5)(**c**)	159(3)	158(3)(**f**)	115(4)	106(3)	105(5)	77(5)	
18 di-TMS	(±)	2998[b]	3029[b]	525(9)	297(**d**)	526(4)	298(27)						
		3006	3037										

[a] 80°C (2 min) then to 160°C (1 min) at 30°C/min and then at 2°C/min to 270°C.
[b] 80°C (2 min) then to 170°C (1 min) at 30°C/min and then at 2°C/min to 270°C.
[c] Mass spectra normalised above *m/z* 40.
[d] Only ions above *m/z* 75 in excess of 2% abundance have been tabulated. Letters (bold) in parentheses denote ions cited in Figs. 7 and 8.

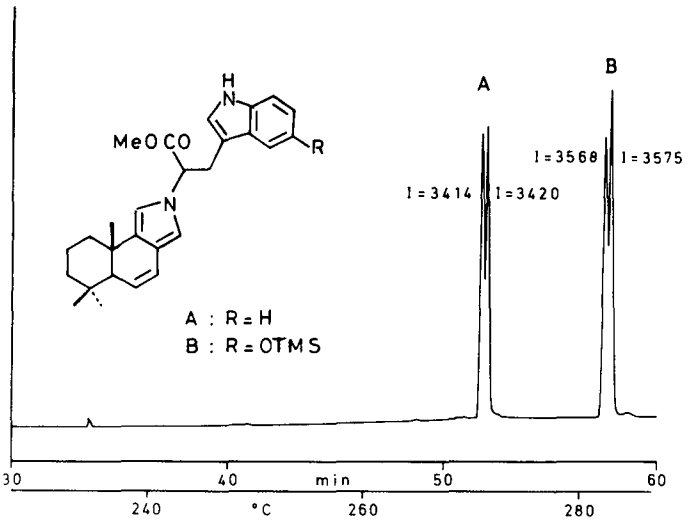

Fig. 4. GC separation of reaction products of (±)-polygodial with (A) (±)-tryptophan methyl ester and (B) (±)-5-hydroxytryptophan methyl ester TMS ether. Column and column temperature programming conditions as in Fig. 3. Me = Methyl.

enantiomers. The separation (ΔI = 5) of peaks of the products from norpseudoephedrine (**13**) was lost in the TMS ethers but recovered (ΔI = 7) in the TBDMS ethers (Table I). However, the *erythro* isomer norephedrine (**14**) afforded pyrrole diastereomers that were separable both in the free and trimethylsilylated forms (Table I). The (+)-amine in each instance (**13** and **14**) yielded, by reaction with (−)-polygodial, the earlier-eluted diastereomers.

The racemic adrenergic agent, methoxamine[9] (**15**), which has the *erythro* configuration, gave diastereomeric products which were exceptionally well resolved as their TMS ethers (ΔI = 24; Fig. 5). This result probably reflects the steric enhancement of conformational energy differences by the *ortho*-methoxyl group. The possibility of a spurious result, due to formation of the *threo* isomer during the reaction, was not regorously excluded, but is considered unlikely. No such epimerisations were observed with any of the related amines; furthermore, achiral reagents yielded single peaks from methoxamine.

(iv) Dihydroxy amines. Three examples of this class were selected: *p*-octopamine (**16**), *erythro*-2-amino-1-phenylpropane-1,3-diol (**17**) and normetanephrine (**18**). Separations of the diastereomeric pyrroles were achieved by means of suitable derivatives (Table I). The di-TMS ethers of the (±)-octopamine derivatives showed ΔI = 7 on both columns.

The diastereomeric derivatives of **17** were partially separated as the 3-mono-TBDMS ethers, but not as the di-TBDMS ethers (Fig. 6). The marked effect of the single TBDMS group at the achiral 3-position may be ascribed to its promotion of intramolecular hydrogen bonding and consequent conformational stabilisation. In the case of normetanephrine (**18**) the di-TMS ethers were adequately resolved.

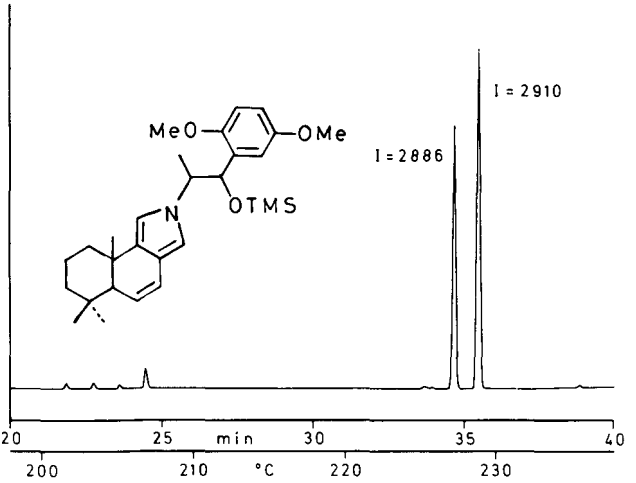

Fig. 5. GC separation of the reaction products of (±)-methoxamine with (±)-polygodial. Column, CP-Sil 5 CB fused-silica capillary (25 m × 0.32 mm I.D.); column temperature, programmed from 80°C (2 min) to 170°C (1 min) at 30°C/min, and then at 2°C/min to 270°C; helium flow-rate, 3 ml/min.

MS properties

The mass spectra showed a variety of notable features. No significant differences were seen between the spectra of the two diastereomers, produced from enantiomeric amines: accordingly, data in Table I are recorded for one of each pair. Molecular ions were observed for every derivative studied (including TBDMS ethers), ranging in relative abundance from 4 to 100%. Other base peaks mainly resulted from expected cleavages, although these were in some cases attended by unpredictable hydrogen

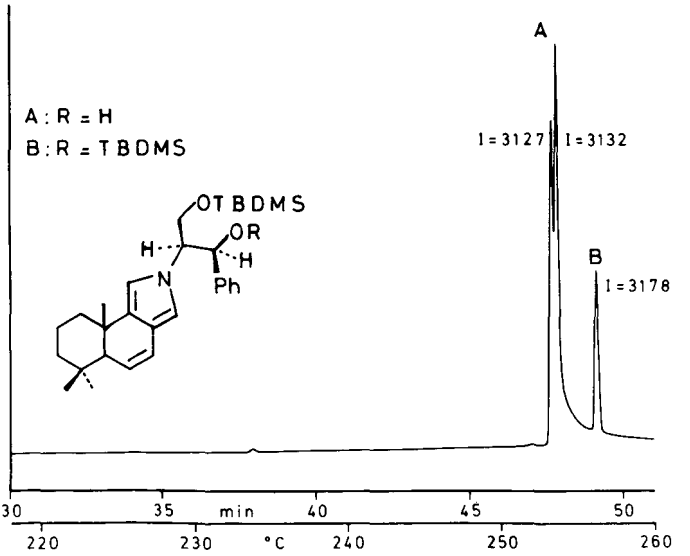

Fig. 6. GC separation of the reaction products of 1*S*,2*S*-(+)-2-amino-1-phenylpropane-1,3-diol with (±)-polygodial as TBDMS ethers. Column and column temperature programming conditions as in Fig. 3.

rearrangements. Likely ion structures for the base peaks (excluding the molecular ions from **4** and **10**) are shown in Fig. 7. The structure **a** relates particularly to parent amine **9**, which contains no methyl group. In the derivative of menthylamine (**5**), (M − 15) ions probably result also from loss of methyl radicals from the monoterpene ring. Of the other ion types, **b**–**e** all ensue from benzylic cleavages, in which the positive charge may be preponderantly on the drimane moiety or on part of the original amine substituent group, according to structural factors. Ion type **f** results from benzylic cleavage, accompanied by loss of C_6H_{12} (84 mass units) from ring A of the polygodial residue. Ions from similar scission of ring A, but mainly of types **g** ([M − 82]) and **h** ([M − 83]) (Fig. 8), representing losses of C_6H_{10} and $C_6H_{11}^{\cdot}$, respectively, were particularly prominent in the mass spectra of derivatives of **4**–**11**. Metastable ions, corresponding to the transition [M]→[M − 82], were observed in this group, except for **4** and **6**. Further salient aspects of the data are outlined below, chiefly in respect of types of ion not mentioned above: some of these are also included in Fig. 8.

(i) Amines lacking other functional groups. The product from tranylcypromine (**4**) gave prominent ions at *m/z* 254, due to loss of a phenyl radical, and at *m/z* 117 ($C_6H_5C_3H_4^+$ resulting from C–N bond scission). Simple C–N cleavage also yields prominent ions —three benzylic cations and one secondary carbonium ion— from derivatives of **6**, **7**, **8** and **9**.

An ion at *m/z* 200, observed in the mass spectra of derivatives of **5**, **8** and **9**, appears to be $C_{15}H_{20}^{+\cdot}$ (**j**), formed by elimination of the pyrrole nitrogen atom together

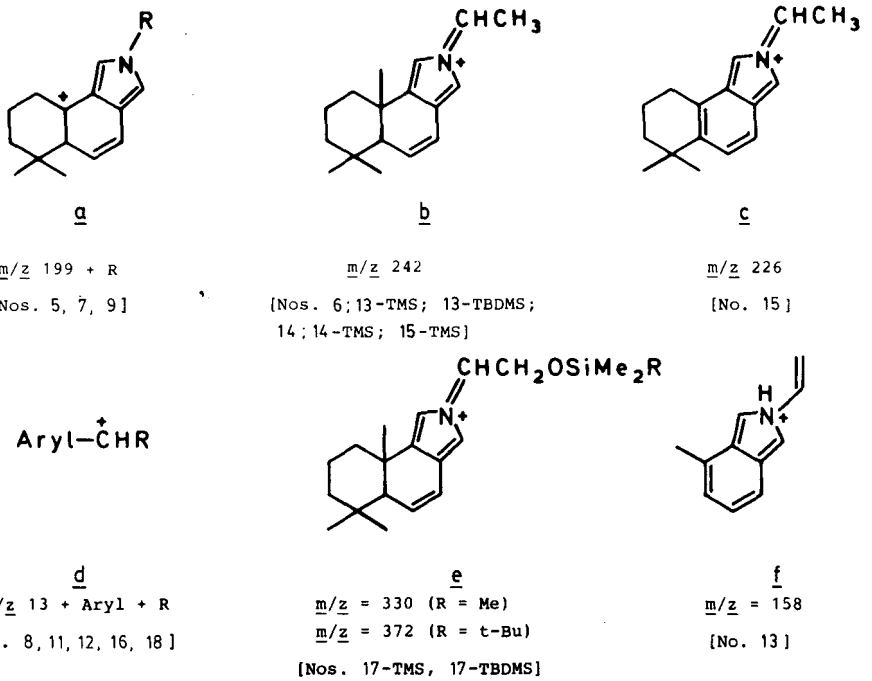

Fig. 7. Ion types constituting the base peaks in the mass spectra (22 eV) of derivatives of the parent amines (excluding the molecular ions of compounds **4** and **10**). Bu = Butyl.

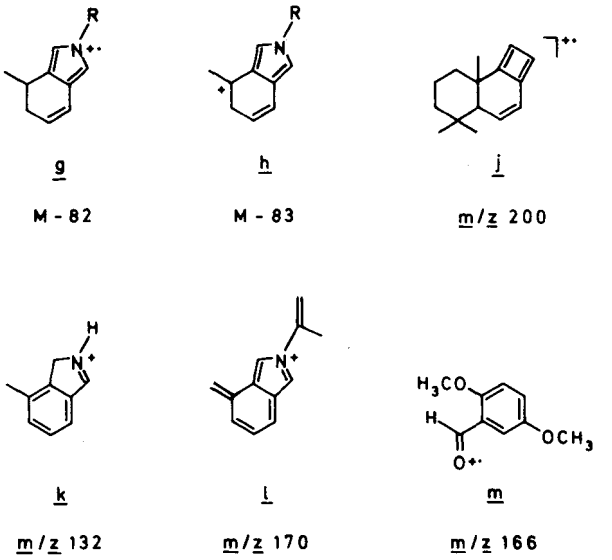

Fig. 8. Postulated structures of some fragment ions.

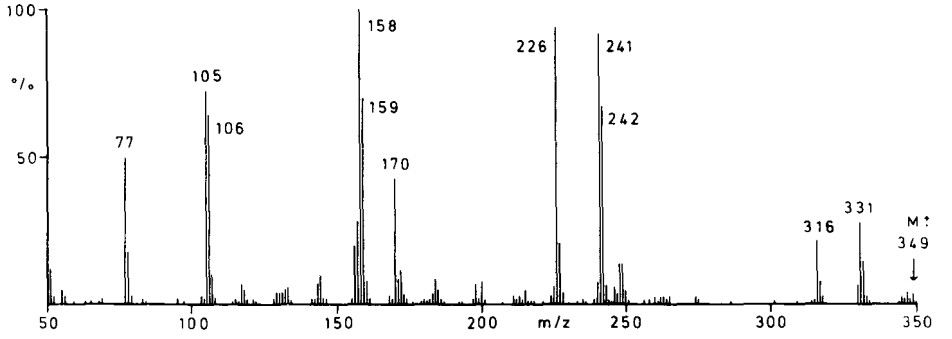

Fig. 9. Mass spectrum (22 eV) of the reaction products of D-(+)-norpseudoephedrine with (±)-polygodial measured on an LKB gas chromatograph–mass spectrometer. Column, DB-1 fused-silica capillary (60 m × 0.32 mm I.D.); column temperature, 240°C; helium carrier and make-up gas flow-rates, 7 ml/min and 25 ml/min, respectively; accelerating voltage, 3.5 kV; filament current, 4A; trap current, 60 μA; source and separator temperatures, 270°C.

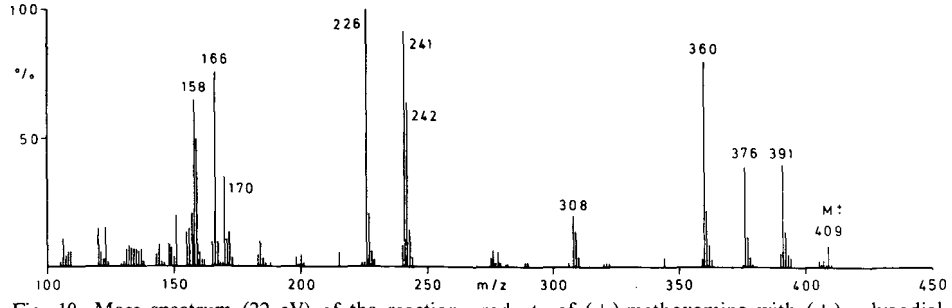

Fig. 10. Mass spectrum (22 eV) of the reaction products of (±)-methoxamine with (±)-polygodial. GC–MS conditions as in Fig. 9.

with its substituent group. An ion of isoindole type (**k**) was also produced, particularly (8–10% relative abundance) by these three derivatives.

The mass spectrum of the derivative of the acetonide (**10**) was of interest in that, besides the expected ions of type [M − 15], types **b**, **c**, **f**, **g** and **h** were all prominent.

(ii) Tryptophan and 5-hydroxytryptophan methyl esters. As expected, the indolyl methyl cations dominated the mass spectra of the derivatives of these compounds (**11** and **12**; the latter being studied as its di-TMS ether), and few other ions were present.

(iii) β-Hydroxy amines. The pyrroles formed from the *threo* and *erythro* isomers norpseudoephedrine (**13**) and norephedrine (**14**) yielded many fragment ions in common but with markedly different relative intensities: thus an ion of type **f** was the base peak in the former spectrum (Fig. 9) but only of 26% relative abundance in the latter (Table I). Ion-radicals of *m/z* 159 were also abundant in both spectra and are undoubtedly analogues of ions **f**. Prominent ions due to benzylic cleavages included $C_6H_5CHO^{+\cdot}$ (*m/z* 106) and $C_6H_5CO^+$ as well as types **b** and **c**. Ions of type [M − H_2O] also occurred. The ion of *m/z* 170 (see also Fig. 10) is attributable to an analogue (type **l**) of ion **f**. Conversion of the pyrrole derivative, derived from norpseudoephedrine, to the TMS or TBDMS ether suppressed most of the fragmentations except for the formation of ions of type **b**, which carried 60–80% of the total ion current.

Methoxamine (**15**) gave a pyrrole showing a very characteristic mass spectrum (Fig. 10) with a variety of prominent ions —thirteen of these having relative abundances of 20% or more. Some of these arose from the presence of the aryl methyl ethers and the benzylic hydroxyl group: thus, sequential losses of H_2O and $CH_3O^{\cdot}$ gave ions at *m/z* 391 and 360 with a strong metastable ion, indicating the latter transition (calcd. 331.5: observed 331.6). Other noteworthy ions were at *m/z* 158 (**f**) and *m/z* 159 (see above); *m/z* 308 ([M − 101]), ascribed to the loss of H_2O and of $C_6H_{11}^{\cdot}$ from the terpenoid moiety (type **h**); and at **m/z** 166, probably representing the dimethoxybenzaldehyde molecular ion *m* (stabilised by the *o*-methoxyl group).

(iv) Dihydroxy amines. In order to obtain satisfactory GC peaks, the condensation products from these substrates were studied as silylated derivatives. The di-TMS ether of the pyrroles formed from **16–18** gave only one major fragmentation, *viz.* the α-cleavage of the TMS ether: however, in the case of the β,β′-dihydroxyamine (**17**) the fragment affording the base peak was not the benzylic ion (*m/z* 179: 6%) but the complementary pyrrole moiety **e** (*m/z* 330: Fig. 7). A metastable ion was present for [M]→[M − 179]. The mono-TBDMS ether of the product from **17** gave a similar result: the only other notable point was the abundance of the molecular ion (36%) compared with [M − 57] (4%), having regard to the general prevalence of the latter type in the mass spectra of TBDMS ethers.

CONCLUSIONS

The qualitative studies reported here for approximately micromolar amounts of reactants show that polygodial undergoes rapid condensation with a wide variety of primary amines to yield stable substituted pyrroles. No interference in the reaction was observed from β-hydroxyl or other hydroxyl substituents: however, where these were present, it was usually advantageous to convert them into TMS or TBDMS ethers to improve their properties in GC.

Among the fifteen chiral amines examined here, five failed to show separation,

by GC, of the derived diastereomeric pyrroles: these are being further studied using more selective phases. In other instances (with silylation of OH or NH groups where appropriate) such diastereomers showed retention index differences (ΔI) ranging from 5 to 24 units. Preliminary work had indicated that chiral aliphatic amines (such as valine methyl ester) did not yield separable diastereomeric pyrroles, and it is noteworthy that all the successful resolutions reported here came from parent amines containing aromatic or heteroaromatic rings.

The mass spectra were satisfactory in yielding clear molecular ions together with a range of informative fragment ions. Several of these ensued from simple and predictable cleavages, but more complex fragmentations also showed regularities that strengthened their value for the characterisation of different amines. One drawback of the present method is that polygodial is not readily available and is somewhat unstable. The former difficulty may be solved if the compound comes into agrochemical use, as partial and total syntheses have been devised. However, other suitably constituted 1,4-dioxo compounds merit investigation, having regard to the speed and experimental simplicity of amine–carbonyl reactions under mild conditions, and particularly to the selectivity of pyrrole formation from primary amines. Preliminary work on the more stable drimenedial, cinnamodial (**1b**)[10,11] has shown that it undergoes similar condensations but that the products are more complex. Further studies are in progress with this reagent.

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CHROMSYMP. 1563

NON-RADIOACTIVE ELECTRON-CAPTURE DETECTOR FOR GAS CHROMATOGRAPHY

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SUMMARY

This paper is an update on the progress made towards developing a practical electron-capture detector which does not require a radioactive source, can be operated in the pulsed mode, and can be used at temperatures up to 800 K. Data obtained using a prototype detector have been reported previously. In this paper results obtained with a "large-volume" but leak-free detector at elevated temperatures are reported, and preliminary data with a practical "small-volume" detector will be discussed. The temperature dependence suggests that the electron-capture mechanisms that have been demonstrated previously are also operative in the non-radioactive detector. The discharge has been examined spectroscopically. Finally, we present characteristic data for the detector under high-purity conditions. These data are similar to those obtained with a radioactive detector and serve as a "base case" for future development.

INTRODUCTION

In a previous publication[1] we presented the rationale for developing a non-radioactive electron-capture detector (ECD) which can be operated at high temperatures and used in the pulsed mode. The original data were obtained on a prototype version of the detector and were obtained to demonstrate the potential of the ionization source. Based on these results, the potential for developing a unique detector for gas chromatography (GC) was good.

We designed a new detector which would simulate a parallel-plate detector and which could be heated to *ca.* 800 K. It was made leak-tight by using flanges. In addition, we examined the spectra of the discharge, using a light pipe. First, the operating parameters of the detector were determined. These data serve as a base case for the non-radioactive detector. Only then were the ECD responses of representative compounds determined as a function of temperature. The general temperature dependence can be used to demonstrate the electron-capture mechanisms when compared with data obtained by using a conventional radioactive ECD. However, in

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the course of these studies, when it became obvious that the "large-volume" detector was too large for good, modern chromatography we redesigned the detector with a smaller volume for use with capillary columns.

It should be emphasized that this detector can be used in both an ionization mode and an electron-capture mode so that it can be both selective and non-selective. Only the electron-capture mode will be discussed in detail in this paper, but some representative chromatograms obtained in the ionization mode will be presented.

EXPERIMENTAL

The description of the basic experimental apparatus has been previously given[1] and will not be repeated. Indeed, the only changes that have been made are in the design of the detectors. The detector shown in Fig. 1 has flanges at each end of the six ports so that leaks are kept to a minimum. Low concentrations of air will affect the performance of the detector when operated with helium in the microwave discharge. If air is introduced into the microwave discharge, the lamp will change from a characteristic peach color to a deep blue or violet. This would result in different excited species and noticeably affects the ionization efficiency. Several ports were built into the detector so that a variety of geometries could be investigated. Because of the number of ports and the bulkier flanges, the volume of this detector is unusually large. However, because of the operation at reduced pressures, the "effective" volume is much lower than in atmosphere operation, due to the increased linear flow-rate. This makes the detector suitable for columns with fewer theoretical plates. However, with small-bore

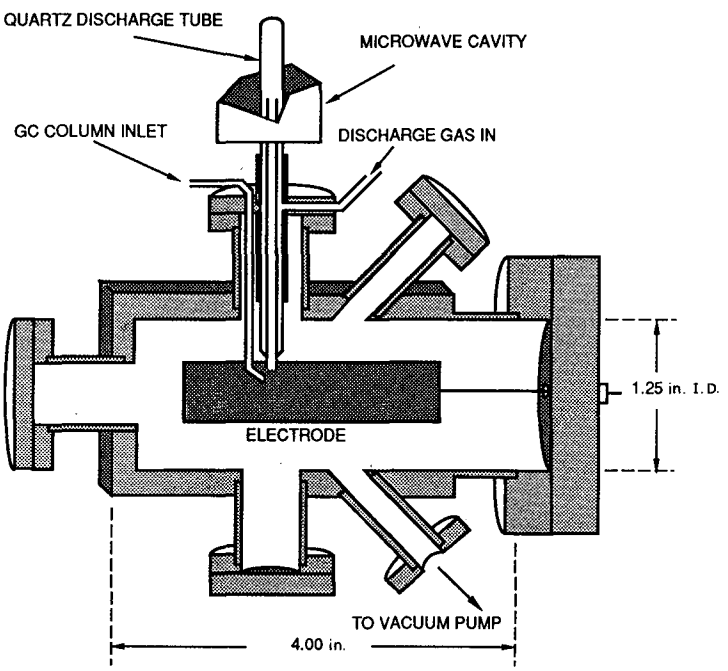


Fig. 1. Schematic diagram of "large-volume" detector.

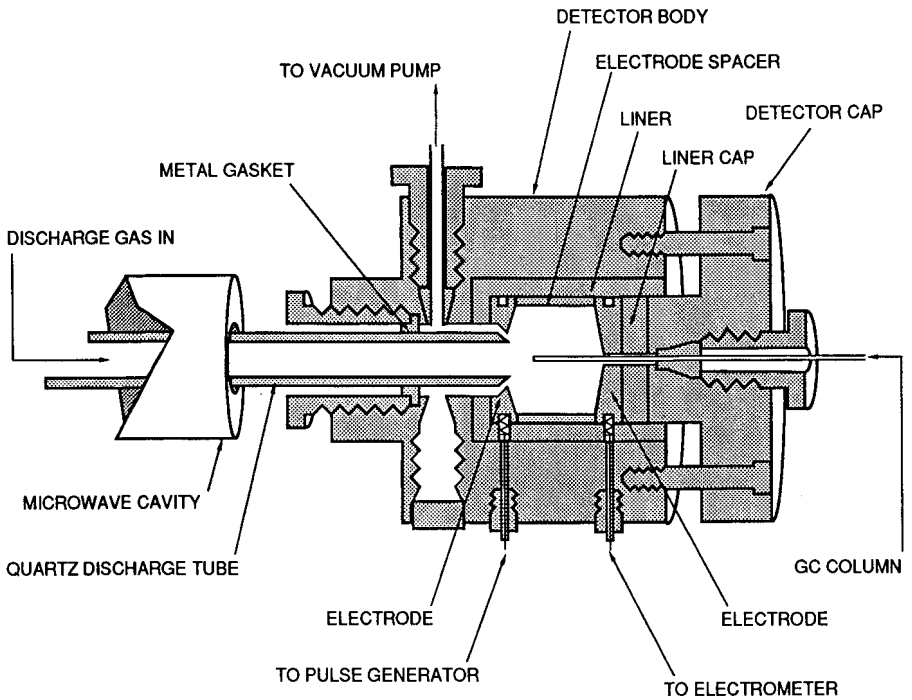


Fig. 2. Schematic diagram of "small-volume" detector.

capillaries, peak broadening was obviously occurring. In addition, the column flow was introduced into the detector at the tip of the quartz lamp so that the helium from the discharge lamp and the column flow were approximately parallel. In this configuration, the two gases might not mix completely during the time spent between the electrodes.

In Fig. 2, we describe a first concept of a smaller volume detector, which is a modification of a commercially available radioactive ECD available from Valco (Houston, TX, U.S.A.). This design incorporates two unique features. First, the capillary column flow is directed opposite to the flow from the discharge lamp, and this apparently leads to good mixing, since the ionization efficiency is greater. Secondly, the parallel-plate arrangement, where the lamp flow enters through the negative electrode, results in no charge collection at the anode unless a potential is applied. Collection of extraneous signals is a common problem with other geometries and also with the ^{63}Ni ECD due to the large range of the β particles and the resulting ionization in the vicinity of the anode. This can result in unusual responses and deviations from the expected concentration dependence. The volume of this detector is 0.64 cm^3. When operated at 40 Torr it has an effective volume of 34 μl, compared to operation at 760 Torr. It appeared that the diameter of the discharge lamp can be reduced considerably, and this will allow further reduction in the volume of the detector. We anticipate the next design to be reduced in volume by a factor of 4 or 5.

RESULTS

The major innovation in the non-radioactive ECD is the discharge source. In order to determine some of the species present in the helium discharge afterglow, we measured the emission spectra of the detector. The emission lines were primarily those of helium metastables. A more detailed report on this work will be given in a paper describing the ionization mode.

In the non-radioactive ECD, the basic ionization reaction can be described as:

$$M^* + CH_4 \xrightarrow{k_p} M + P^+ + e^{-*} \tag{1}$$

$$e^{-*} + CH_4 \longrightarrow e^- \text{ (thermal)} + CH_4 \tag{2}$$

where the overall production rate of thermal electrons is given by k_p. The thermal electrons can then recombine with positive species according to

$$e^- + P^+ \xrightarrow{k'_D} \text{neutrals} \tag{3}$$

In the presence of a capturing species, AB, the reactions that take place are

$$AB + e^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB^- \xrightarrow{k_2} A + B^- \tag{4}$$

$$AB + e^- \xrightarrow{k_{12}} A + B^- \tag{5}$$

$$N^- + P^+ \xrightarrow{k'_N} \text{neutrals} \tag{6}$$

In these reactions, M* represents any helium metastable (He^*, He^+, He_2^+, He_2^*), P^+ is any positive ion, and N^- is any negative ion. The rate constants are indicated above and below the respective reactions. This model is the same as that used for the analysis of the radioactive ECD, and the current studies serve to investigate possible differences in the two detectors[3].

Based on this model, the ECD response, expressed as K, can be obtained in terms of the various rate constants as follows

$$\frac{b - [e]}{[e]}\left\{k'_D[P^+]\right\} = K[AB] = \left\{\frac{k_1(k_2 + k'_N[P^+])}{(k'_N[P^+] + k_{-1} + k_2)} + k_{12}\right\}[AB] \tag{7}$$

where it is assumed that the extent of capture is small enough that [AB] and $[P^+]$ are essentially constant.

In our preliminary study, operational curves for various parameters were obtained and the similarity between the non-radioactive and the radioactive ECDs

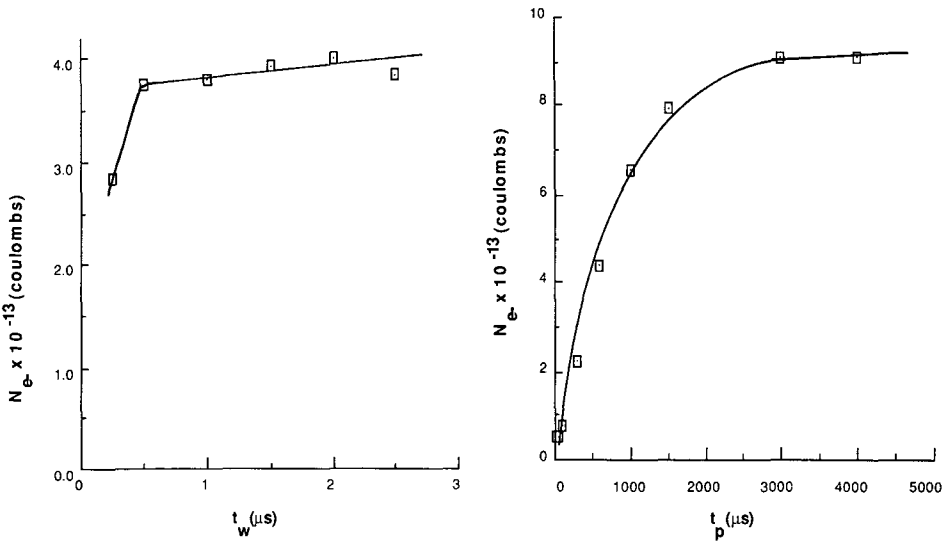


Fig. 3. Number of electrons collected per pulse (N_e) *vs.* pulse width (t_w): T = 500°C; pressure P = 29 Torr; power, 50 W; voltage, 50 V; t_p = 100 μs.

Fig. 4. Number of electrons collected per pulse (N_e) *vs.* pulse period (t_p): T = 200°C; P = 28 Torr; power, 50 W; voltage, 50 V; t_w = 2 μs.

were noted. We have obtained improved data, using the leak-free detector, and find that the abnormalities observed in the previous study were artifacts. To illustrate these results, we include plots of N_e (number of electrons per pulse) *vs.* t_w (pulse width) and N_e *vs.* t_p (pulse period) in Figs. 3 and 4.

The variation of N_e with t_w shows a plateau at *ca.* 0.5–1.0 μs, which is more like the radioactive ECD. In addition, the slope in the plateau region is lower than reported previously[1,2] and is more like that found with the radioactive ECD.

The variation of N_e with t_p at temperature T = 200°C is more like that obtained with a "clean" radioactive ECD, where the plateau is reached at 3000 μs. The effect of impurities on such a curve can be seen in Fig. 5 where data with and without a column are given. With the column, the plateau is reached at about 1500 μs, and the magnitude is reduced by a factor of 3. This column is a "bonded-phase" silica capillary column, where the stationary phase contains polytrifluoromethylsiloxane groups. Apparently, the "bleed" from the column consists of compounds containing the trifluoromethyl group, which cause the reduction in the standing current at long-pulse intervals. Presently, we are using a non-polar bonded-phase silica capillary column, where the stationary phase is polydimethylsiloxane and the effect of the "column bleed" on the non-radioactive ECD is reduced considerably.

These curves can be used to obtain values of $k_p V_r$ (V_r is the reaction volume) and $k'_D[P^+]$, since the initial slope = $k_p V_r$ and $N_e(\max) = k_p V_r / k'_D[P^+]$. This has been done as a function of temperature, and the results are given in Figs. 6 and 7. The experiments were performed without a column and with and without methane adjustment. It is believed that the methane adjustment compensated for the temperature effect on the

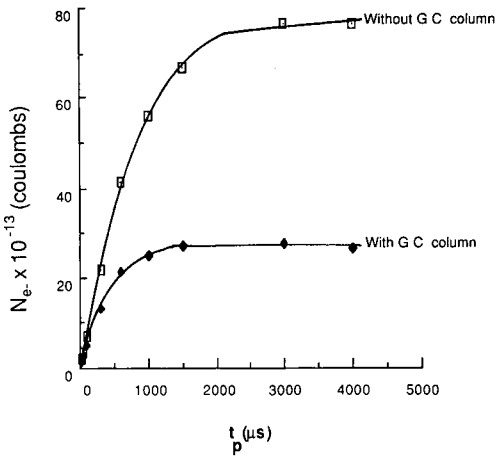
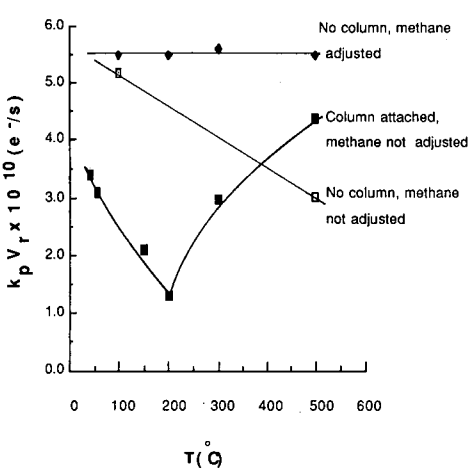


Fig. 5. Number of electrons collected per pulse (N_e) *vs.* pulse period (t_p), with and without the GC column: T = 100°C; voltage, 50 V; t_w = 2 μs; P = 28 Torr; power, 50 W (without), 58 W (with). Column, 10 m × 0.32 mm I.D. cross-linked methylsilicone.

reaction volume. Thus, Fig. 6 shows that by adjusting the methane concentration, the value of k_pV_r could be made constant. However, as shown in Fig. 7, there is an increase in the quantity $k_D = k'_D[P^+]$ with increasing temperature. The data for the system with the column are given to show the effect of column bleed on the various parameters. Of course, the results are a function of the specific column being used and its prior history.

The normal parameters of interest to those using the ECD for analytical purposes are the minimum detectable quantities and the linearity of the response. In Fig. 8 linearity plots are given for several compounds. Note that the units on the *x*-axis are femtomoles.



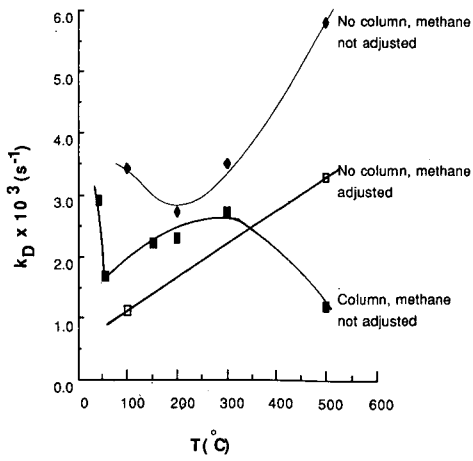


Fig. 6. Electron production (k_pV_r) *vs.* temperature: P = 28 Torr; power, 50 W; voltage, 50 V; t_w = 2 μs.

Fig. 7. Pseudo first order recombination rate constant (k_D) *vs.* temperature: P = 28 Torr; power, 50 W; voltage, 50 V; t_w = 2 μs.

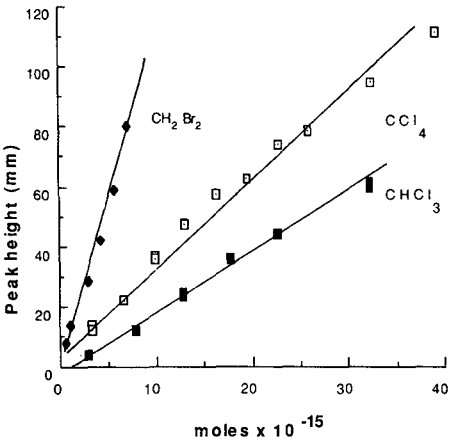


Fig. 8. Concentration dependence for CCl_4, CH_2Cl_2 and CH_2Br_2: P = 23 Torr; power, 12 W; voltage, 50 V; t_w = 2 μs; t_p = 500 μs.

In our previous paper, we gave representative values of the minimum detectable amounts at room temperature. It is also important to know the temperature dependence of the response in order to optimize the detector temperature. For example, for the analysis of compounds that have an activation energy for dissociation, a higher temperature is better. On the other hand, for compounds that form stable negative ions, a lower temperature is better. In addition, there are some compounds, such CCl_4, where the response is independent of temperature. We have determined the temperature dependence for CCl_4, CH_2Cl_2 and C_6F_6. The data are shown in Fig. 9. The general temperature dependence of the capture coefficient is as

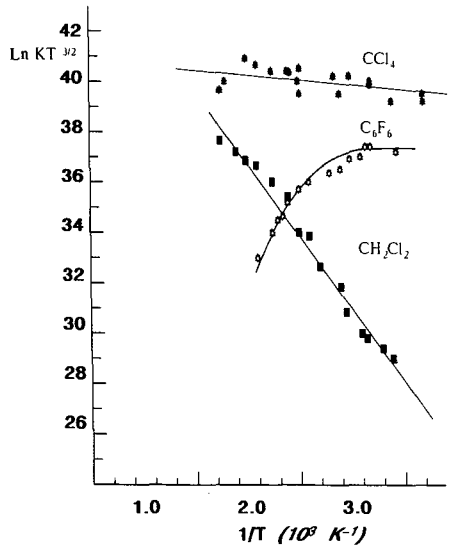


Fig. 9. Temperature dependence for ECD response of CCl_4, C_6F_6 and CH_2Cl_2.

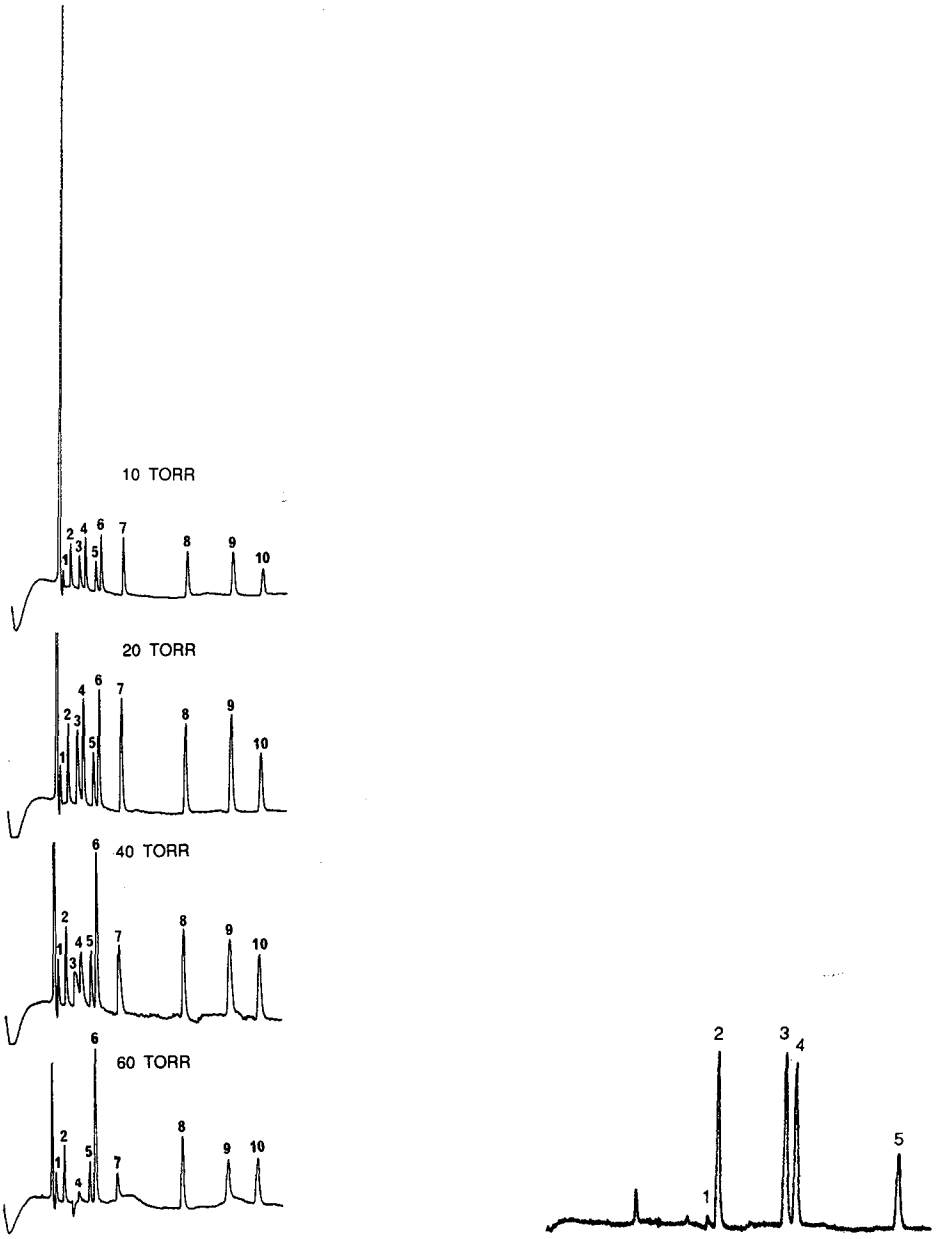


Fig. 10. Ionization mode chromatograms for the "small" detector. Peaks: 1 = C_3H_8; 2 = *n*-C_4H_{10}; 3 = CH_3CN; 4 = 2-C_3H_7OH; 5 = CH_2Cl_2; 6 = CS_2; 7 = C_4H_8O (methyl ethyl ketone); 8 = C_6H_6; 9 = $C_6H_{14}O$ (*n*-propy' ether); 10 = *n*-C_7H_{16}. The amounts of each component are about the same: 5–10 ng. Column, 30 m × 0.32 mm I.D., 1 μm RSL-150 bonded phase; *T*(column) = 40°C; carrier gas, 3 ml/min helium; discharge gas, helium; discharge power, 100 W; pulse width, 3 μs; t_p = 30 μs; pulse voltage, 80 V; *T*(column) *ca.* 70°C.

Fig. 11. ECD mode chromatogram for the "small" detector. Peaks: 1 = CH_2Cl_2, 20 ng; 2 = CS_2, 5 ng; 3 = C_6F_6, 0.03 ng; 4 = $CHCl_3$, 0.6 ng; 5 = CCl_4, 0.003 ng. All other conditions are the same as in Fig. 10, except for the addition of methane; t_p = 300 μs; *P* = 40 Torr.

expected. Thus, the minimum detectable amount for CH_2Cl_2 is decreased to the fmol range at 500°C, while that for C_6F_6 is increased to the pmol range.

On the basis of these results, it is clear that the discharge source is suitable for use as a GC detector. However, we also discovered that the volume of the detector was seriously degrading the chromatographic performance with extra-column broadening. Thus, we have redesigned the cell with a smaller reaction volume.

We have demonstrated that this detector can be used with capillary columns in the ionization mode. Shown in Fig. 10 are a series of chromatograms, obtained with the "small detector" as a function of detector pressure. In this case, the response is the result of an increase in current due to the ionization of the eluting compound. The non-selective nature of the detector is apparent, since the amount of each component is comparable (5–10 ng). Note that the relative responses change when the cell pressure is changed. The response for CS_2 increases as the pressure is increased while that for the other peaks reach a maximum at 20 Torr and then decrease as the pressure is increased. The percent ionization for heptane is 0.14% in the "small"-volume detector.

The new detector was operated in the ECD mode by adding methane make-up to the helium carrier gas just prior to the detector. The methane concentration and pressure were adjusted to maximize the response for CCl_4 and CS_2. As the pressure and methane concentration increase, the percent capture for both CS_2 and CCl_4 increases. This pressure sensitivity is probably due to thermalization of the electrons. At higher pressures, smaller amounts of methane were required to produce the same response. At 40 Torr the maximum response could be obtained while maintaining a satisfactory standing current. The results shown in Fig. 11 were obtained under these conditions. The response shown is for 3.2 pg or 21 fmol of CCl_4. The other compounds in the mixture show less sensitivity in the ECD, as can be seen from the amounts of each compound injected, which are given in the legend. In Fig. 11, the response is a measure of the decrease in the standing current.

The noise, N, which we have observed comes primarily from the microwave power supply and is of two types: one is periodic with *ca.* 30 Hz frequency of *ca.* $4 \cdot 10^{-12}$ A and the other a random noise level of *ca.* $1 \cdot 10^{-12}$ A. Using $2N$ as the detectability, the value for CCl_4 is 128 fg, based on the periodic noise, and 32 fg, based on the random noise. These values are comparable to the 68 fg reported in our earlier paper[1].

CONCLUSIONS

Based on the data obtained from the "leak tight" detector, the operational parameters of the non-radioactive ECD are similar to those of the radioactive ECD. Based on the temperature dependence of the ECD response, the mechanism for ECD response is the same in the non-radioactive and the radioactive ECDs. This clearly demonstrates that the microwave source can be used in an ECD for GC.

The small-volume detector has been demonstrated to be a practical detector for GC. In the ionization mode, it is a non-selective detector with an ionization efficiency for heptane of 0.14%. In the electron-capture mode, it has the typical high sensitivity for CCl_4. Future work will center on the optimization of the responses.

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ANALYTICAL-SCALE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OMEGA-3 FATTY ACID ESTERS DERIVED FROM FISH OILS

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SUMMARY

Fish oil triglycerides were transesterified to their corresponding methyl and ethyl esters. The esters were separated by reversed-phase high-performance liquid chromatography on a 10-cm column of 5-μm octadecylsilyl silica, using a ternary mobile phase of acetonitrile–tetrahydrofuran–water (9:5:11). The separation of principal saturated and unsaturated C_{14}–C_{22} fatty acid esters was accomplished in 60 min. For the polyenoic acid esters, detection limits of *ca.* 26 μg were observed, and baseline resolution of the important critical pair of esters of the ω3 fatty acids, eicosapentaenoic acid (C20:5ω3) and docosahexaenoic acid (C22:6ω3) was achieved. However, to obtain baseline resolution of the esters from minor fatty acid esters, a urea crystallization procedure was required prior to chromatographic analysis. The influence on the reversed-phase chromatographic retention of four different ester moieties on the fatty acids was also studied. The *n*-alkyl esters exhibited an increase in ln k' with increasing chain length and with increasing lipophilic character of the alkyl ester moieties; however, with the aromatic benzyl derivatives there was a decrease in ln k' compared with the alkyl esters.

INTRODUCTION

Interest in ω3 farry acids was initiated by results of epidemological studies performed on Greenland Eskimos. These peoples experienced a low incidence of death from ischemic heart disease (5.3%) compared to Western populations (39.7%)[1]. Further studies[2–8] attribute this low mortality figure and many other health benefits to a diet high in ω3 fatty acids. These acids are long-chain fatty acids which have methylene interrupted double bonds and the last double bond three carbons from the methyl terminus. These particular fatty acids occur naturally at high concentrations in the body oils of cold water fish (*i.e.*, sardine, menhaden, anchovy). The major fatty

[a] The shorthand notation C*n*:*x*ω*y* will be utilized throughout this manuscript. The *n* denotes the carbon chain length, *x* the number of double bonds, and *y* the location of the last double bond in relation to the terminal methyl group of the fatty acid.

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acids of interest are eicosapentaenoic acid (C20:5ω3 or EPA) and docosahexaenoic acid (C22:6ω3 or DHA)[a].

At present, the method of choice for the analysis of complex mixtures of polyenoic fatty acids such as those derived from fish oils, is capillary gas chromatography (GC) with pre-chromatographic derivatization and mass spectrometric (MS) detection. However, GC is impractical for the purification of the large amounts of polyenoic fatty acids required for biological and clinical studies. Moreover, the temperatures required in GC may cause degradation of oxidized long-chain polyunsaturated fatty acids which are present as minor components of the mixture.

In the past fifteen years, complex mixtures of these polyunsaturated long-chain fatty acids or their *n*-alkyl esters contained in natural and modified products, such as margarines[9], vegetable oils[10] and evening primrose oils[11] have been successfully separated by reversed-phase high-performance liquid chromatography (RP-HPLC). Under the relatively mild conditions of RP-HPLC, little degradation of polyenoics occur, and methods can be easily scaled-up for semi-preparative or preparative use. Because methylene-interrupted polyunsaturates do not have strongly absorbing chromophores in the UV region, detection by refractive index or far-UV detection (205–214 nm) has been utilized in RP-HPLC separations of free fatty acids and their aliphatic esters. Refractive index detection is less sensitive than UV detection. However, with far-UV detection solvents absorbing UV below 210 nm cannot be used. The RP-HPLC separations reported to date have generally involved derivatization designed to incorporate aromatic chromophores allowing detection by fluorescence[12,13] or UV[14,15] detection. Thus, relatively low levels of fatty acids may be detected in complex sample matrices, such as biological fluids.

Our goal was to develop a RP-HPLC procedure for the analysis of ω3 fatty acid esters which could eventually be adapted to the preparative isolation of quantities of EPA and DHA, suitable for use in nutritional or biochemical studies. In order to accomplish our objective, the factors involved in sample storage, the influence of the ester moiety on retention behavior, and pre-chromatographic procedures were evaluated and the mobile phase for each chromatographic condition was optimized.

EXPERIMENTAL

Materials

Standards of fatty acid methyl and ethyl esters and trimyristin were purchased from Nu-Chek Prep (Elysian, MN, U.S.A.). All solvents used for extraction, transesterification and chromatography were obtained from Fischer Scientific (Springfield, NJ, U.S.A.). Mobile phases were filtered through 0.45-μm Nylon-66 membrane filters (Rainin Instruments, Ridgefield, NJ, U.S.A.), and were degassed by saturation with helium. Butylated hydroxytoluene (BHT) was obtained from Aldrich (Milwaukee, WI, U.S.A.).

Sardine oil was obtained from the commercially available dietary fish oil capsules Promega™ (Parke-Davis Consumer Health Products Group, Morris Plains, N.J., U.S.A.), menhaden from MaxEPA™ (Seven Seas Health Care, Hull, U.K.) and cod liver oil from Dale Alexander™ Cod Liver Oil (Twin Labs., Ronkonkoma, NY, U.S.A.).

Analytical HPLC

The chromatographic system consisted of an M600 pump, equipped with a U6K injector and a R401 differential refractometer, operated at ambient temperature (Waters Division of Millipore, Milford, MA, U.S.A.). The separations were performed on a Whatman ODS-3 RAC II column (100 × 4.6 mm I.D., 5-μm particle size) (Whatman, Clifton, NJ, U.S.A.). A guard column (30 × 4.6 mm I.D.) was dry-packed with Supelcosil LC-18 40-μm material (Supelco, Bellefonte, PA, U.S.A.). The esters were eluted with a mobile phase of acetonitrile–tetrahydrofuran (THF)–water (9:5:1 1, v/v/v) at a flow-rate of 2.0 ml/min. Data were recorded on an Omniscribe stripchart recorder (Houston Instruments, Austin, TX, U.S.A.).

Stock solutions of ester standards were prepared at concentrations of 100 mg/ml. The diluent was peroxide-free THF containing 0.005% (w/v) BHT. The standards were stored in Reactivials™ (Supelco) blanketed with nitrogen at 4°C. Stored in this manner, the standards were stable for many months. Aliquots of samples or standards were injected with a 100-μl syringe.

Base-catalyzed transesterification

Transesterification of triacylglycerols was accomplished rapidly and quantitatively via reaction with the respective sodium alcoholate–alcohol. The procedure of Christie[16] was used with minor modifications. Typically, the fish oil in one capsule (1 g) was removed with a syringe and immediately dissolved in 20 ml of peroxide-free THF. A 40-ml volume of 0.5 *M* sodium methoxide, sodium ethoxide, sodium *n*-butoxide or sodium benzoxide, freshly prepared by dissolution of freshly cut sodium metal in the corresponding alcohol, was added. After 10 min at 5°C, the reaction was quenched by the addition of 2 ml of glacial acetic acid, followed immediately by the addition of 100 ml of distilled water. The esters were extracted twice with dietyl ether (300 ml) and the ether layer was dried over anhydrous sodium sulfate containing 10% potassium hydrogencarbonate. The resulting slurry was filtered and the remaining solvent was removed under reduced pressure. The residue was dissolved in 1 ml of peroxide-free THF, containing 0.005% (w/v) BHT. Aliquots of solutions were injected into the chromatographic system.

Recovery experiments

In order to confirm the quantitative conversion of the triglycerides to the corresponding esters, 85 mg of the trimyristin was transesterified to methyl myristate in two separate experiments, using the procedure described. An aliquot of the transesterified trimyristin was analyzed chromatographically and quantified by comparison to a methyl myristate standard curve.

Pre-chromatographic procedures

The urea crystallization procedure of Gunstone *et al.*[17] for methyl esters was used with modifications. Following transesterification, the ethyl esters were dissolved in 8 ml of ethanol which contained urea (250 mg urea/ml). The crystallization was initiated at room temperature and then proceeded for 24 h at 4°C. After the solution had thawed, the resulting crystals were washed with urea-saturated ethanol. The ethyl esters were extracted twice from the filtrate with 50 ml of diethyl ether. The remaining solvent was removed by rotary evaporation and the ethyl esters were dissolved in 2 ml

of peroxide-free THF, containing 0.005% (w/v) BHT. Recovery experiments indicated yields after recrystallization were 83% for the EPA ester and 79% for the DHA ester, repectively; yields were in agreement with those reported in the literature[17].

The low-temperature crystallization procedure described by Gunstone *et al.*[17] was applied, unmodified,to both the methyl and ethyl esters.

To minimize degradation of the fish oil esters, methods of storage were evaluated. A sample of fish oil triglycerides was transesterified and the resulting esters were divided into four aliquots: aliquot 1 was dissolved in peroxide-free THF which contained 0.005% BHT (w/v) as an antioxidant, blanketed with nitrogen and stored in a freezer (control sample); aliquot 2 was kept under the same conditions but without BHT; aliquot 3 was not blanketed with nitrogen; aliquot 4 was kept at room temperature in the light.

Column washing procedures

After many weeks of daily operation, the back pressure of the chromatographic system increased but no loss of chromatographic performance was noted. The increase in back pressure was presumably due to the slow precipitation onto the column packing of very-long-chain fatty acids (C_{24} and longer). These acids have a limited solubility in the mobile phase. Therefore, both the analytical and guard columns were washed overnight with degassed, peroxide-free THF at 0.2 ml/min to removed any fatty acid residue. The next day, the column back pressure was normal. After equilibration, the column plate count was checked with the C14:1ω7, C16:1ω7 and C18:1ω9 methyl ester standards.

Physical methods of identification

The ester functionality of benzyl myristate generated by our method was confirmed by IR spectroscopy. The C14:0 benzyl ester, which was collected as it was eluted from the column, was extracted into chloroform. The spectrophotometer employed was a Model FTS-40 Fourier transform IR spectrometer (Digilab, Boston, MA, U.S.A.). Spectral data at 4 cm^{-1} resolution were acquired at 256 scans per file. The spectral window region was 3300–800 $^{-1}$. The sample was analyzed as a thin film, deposited on potassium bromide plates. Peak identification of the C20:5ω3 methyl ester was confirmed by GC–MS by comparison with a standard MS spectrum.

RESULTS AND DISCUSSION

Chromatography

The chromatographic profiles representing saturated, mono-, di- and poly-unsaturated long-chain fatty acid esters derived from marine triacylglycerols, were very complex. Miwa *et al.*[18] were the first to introduce the equivalent chain length (ECL) theory [ECL = (No. of carbon atoms in chain) -2 (No. of bonds)] of retention of long chain fatty acids in chromatographic systems. In general, our data support the ECL,theory; *i.e.* polyunsaturates are eluted before saturates of equal chain length, and shorter-chain before longer-chain fatty acid esters. Compounds with the same ECL are known as critical pairs. In order to obtain the maximum resolution for the critical pairs of long-chain fatty acid esters derived from fish oil, the effect of changing the aqueous content or ratio of organic modifiers (acetonitrile–THF) in the mobile phase was

investigated. Since it has been observed that there is increased selectivity with binary and ternary mobile phases containing THF[9,19] the relationship between ln k' and acetonitrile–THF ratio for the methyl esters of two critical pairs, C20:5ω3 and C22:6ω3 (ECL = 10) and C16:0 and C18:1ω9 (ECL = 16) methyl esters was determined (Fig. 1). The resolution of C20:5ω3 and C22:6ω3 is better than that of the more saturated fatty acid esters with a higher ECL number. Our results are in accordance with those of Baile *et al.*[9] and Tanaka *et al.*[19], who found that the difference in the free energy of interaction ($\Delta G = RT \ln k'$, where R is the universal gas constant and T temperature) between the polyunsaturated critical pair C20:5ω3 and C22:6ω3 is greater than that of the more saturated critical pair C16:0 and C18:1ω9.

Representative chromatograms of fatty acid methyl esters, derived from three fish species most frequently used in dietary supplements, are shown in Fig. 2. In the chromatograms, the number and types of fatty acid methyl esters appear to be the same. However, ratios of the amounts of methyl esters of EPA to DHA differ according to the species of fish. The elution order of the identified fatty acid methyl esters is: C20:5ω3 (ECL = 10)> C14:0 (ECL = 14)> C16:1ω7 (ECL = 14)> C22:6ω3 (ECL = 10)> C18:2ω6 (ECL = 14)> C16:0 (ECL = 16)> C18:1ω9 (ECL = 16). The trend is generally as predicted by the ECL theory, but the C22:6ω3 (ECL = 10) methyl ester is eluted after the C14:0 (ECL = 14) and the C16:1ω7 (ECL = 14) esters. The peaks of the fatty acid methyl esters were identified by comparison of retention times in chromatograms of commercial standards. Peak identification for the C20:5ω3 and C22:6ω3 esters was confirmed by GC–MS.

The reproducibility of retention for the seven identified fatty acid esters was determined by replicate injections of standard compounds. The reproducibility (relative standard deviation) of retention ranged from 0.6 to 4.7% R.S.D. for the methyl esters and from 0.7 to 7.0% R.S.D. for the ethyl esters. The lower limit of detection (LLD), which was defined as the signal-to-noise ratio (S/N) = 2, was 26 μg for the polyenoic acid esters and higher for the saturated and monosaturated acid esters C14:0, C16:0 and C18:1ω9, where the LLD was 42, 86 and 67 μg, respectively.

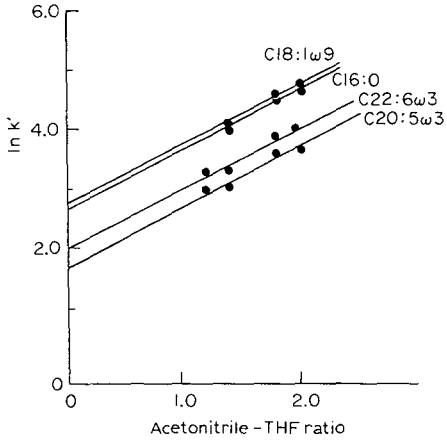

Fig. 1. Mobile phase study: plot of ln k' *versus* acetonitrile–THF ratio. Critical pair 1: C18:1ω9 and C16:0 methyl esters (ECL = 16). Critical pair 2: C22:6ω3 and C20:5ω3 methyl esters (ECL = 10).

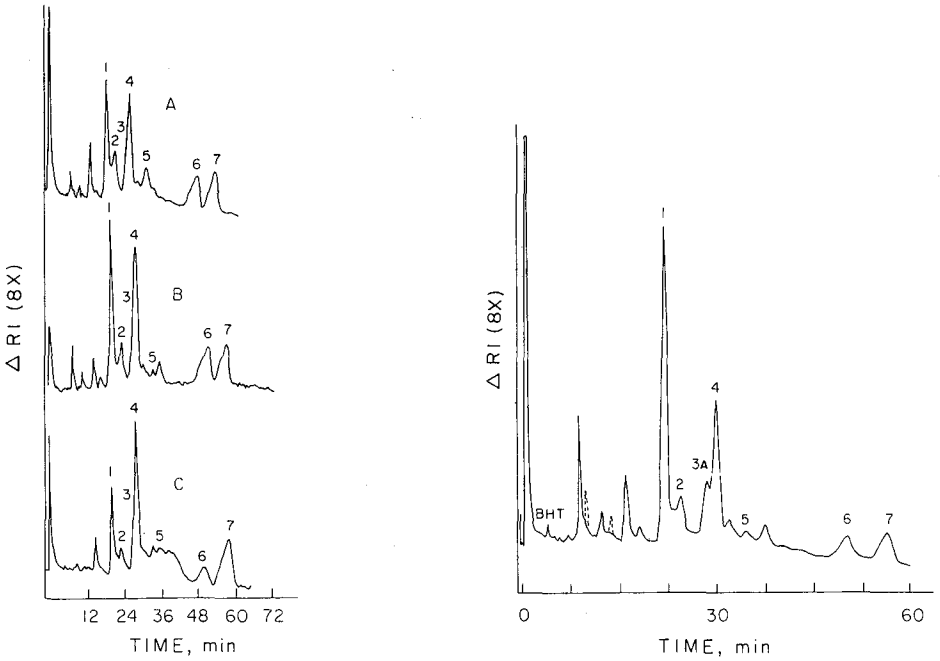

Fig. 2. RP-HPLC of methyl esters, derived from three different fish oil sources: sardine (A), menhaden (B) and cod liver (C). Identified fatty acid methyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω9; (4) C22:6ω3; (5) C18:2ω6; (6) C16:0; (7) C18:1ω9; BHT is eluted right after the void volume; remaining peaks have not been positively identified. Mobile phase: acetonitrile–THF–water (9:5:11) at 2.0 ml/min; stationary phase, Whatman ODS-3 RAC II (100 × 4.6 mm I.D.); detection, refractive index at ambient temperature.

Fig. 3. Degradation study: RP-HPLC of aliquot 2 (no BHT). Analysis day 1, – – –; day 21, ———. For identified methyl esters in order of their elution and chromatographic conditions see Fig. 2.

The linearity of detector response was confirmed for all the fatty acid methyl esters in the range of 100 to 1000 μg.

Storage conditions

Methylene-interrupted polyunsaturated long-chain fatty acids and their esters are susceptible to autoxidation. After 21 days without BHT, a new peak, peak 3A, which was not identified, was present (Fig. 3). In addition two peaks prior to peak 1, which were seen in the chromatogram of the control sample were no longer present (Fig. 3). These peaks were also absent from the aliquots that were not stored under a blanket of nitrogen (aliquot 3) or were kept at room temperature in the light (Aliquot 4). Therefore, throughout our studies, all samples and standards were stored at 4°C in the dark as a solution in peroxide-free THF, containing 0.005% BHT and blanketed with nitrogen. BHT is eluted near the void volume in the chromatogram and does not interfere with the analysis of the long-chain fatty acid esters.

Although the rate of presumed free radical reactions are slowed by the precautions taken in the handling and storage of the fatty acid esters, after six months there was evidence that some of the polyunsaturates were degraded. However, no sign of degradation of EPA and DHA was observed during our work.

Pre-chromatographic procedures

Baseline separation of the critical pairs was achieved when the low-temperature fractionation procedure was used prior to chromatography to diminish the content of the more saturated long-chain fatty acid esters. However, C16:1ω7, which was eluted together with DHA, and C14:0, which was not completely separated from EPA, were not removed by this procedure. The procedure was also applied to decrease substantially the more saturated triglycerides in fish oils, to concentrate triglycerides before transesterification, or to concentrate the fatty acid methyl esters. However in the Promega chromatograms there was no difference in the chromatograms of fatty acid esters before and after low temperature crystallization. Probably, the sardine oil used in these capsules had been concentrated by the low-temperature procedure during processing.

The urea crystallization procedure was successfully utilized to remove a significant amount of the more saturated fatty acid esters. Fig. 4 represents a chromatogram of the sardine oil esters after urea crystallization. This chromatogram clearly shows the prominence of the major omega-3 C20:5ω3 (1) and C22:6ω3 (2) ethyl esters.

Ester moieties

The retention behavior of the methyl, ethyl, *n*-butyl and benzyl esters of six long-chain fatty acids, derived from the sardine oil source, are represented graphically in Fig. 5 as ln *k' versus* the carbon number of the ester moiety. The curves from bottom to top for the following fatty acid esters represent in order of elution: (1) C20:5ω3; (2) C14:0; (3) C22:6ω3; (4) C18:2ω6; (5) C16:0; (6) C18:1ω9. For the aliphatic moieties, retention increases with increasing carbon-chain length[20,21], *i.e.*, retention increases with increasing lipophilicity. However, the aromatic benzyl esters of the fatty acids are less retained than their corresponding methyl esters. This behavior can be attributed to steric hindrance of the more bulky benzyl derivatives than of the *n*-alkyl esters. The polarizability of the benzyl derivatives may also cause a greater affinity for the polar mobile phase. Thus, they are less retained than the shorter, less polarizable *n*-alkyl esters.

Representative chromatograms of the four types of derivatives of the fatty acids in sardine oil are shown in Figs. 2A and 6–8: Fig. 2A, methyl esters; Fig. 6, ethyl esters;

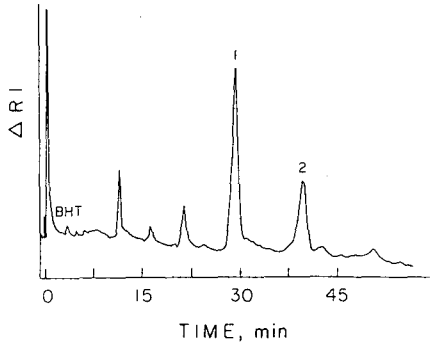

Fig. 4. RP-HPLC of sardine oil long-chain fatty acid ethyl esters after urea crystallization. Identified fatty acid ethyl esters in order of their elution: (1) C20:5ω3; (2) C22:6ω3. For chromatographic conditions see Fig. 2.

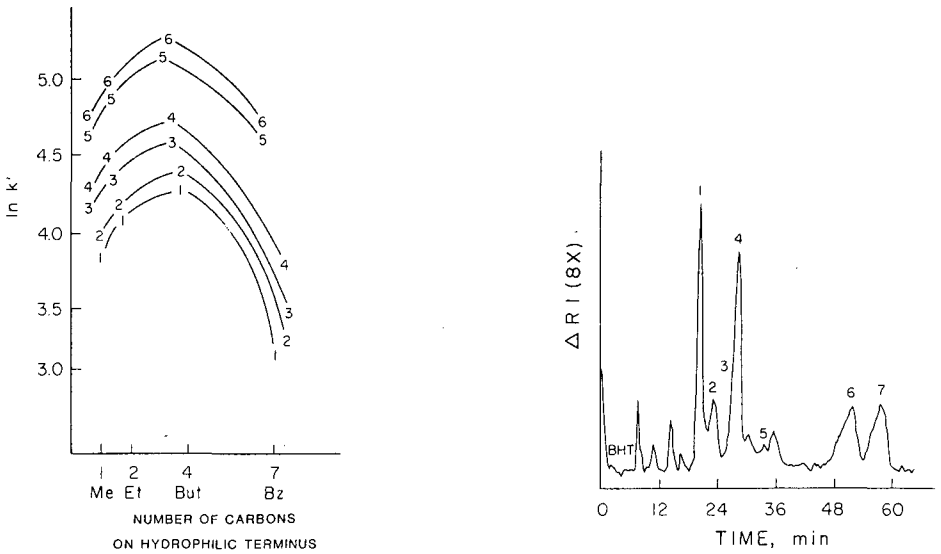

Fig. 5. Plot of ln *k' versus* carbon-chain length of methyl (Me), ethyl (Et), *n*-butyl (Bu) and benzyl (Bz) esters: (1) C20:5ω3; (2) C14:0; (3) C22:6ω3; (4) C18:2ω6; (5) C16:0; (6) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 6. RP-HPLC of ethyl esters of long-chain fatty acids, derived from sardine oil. Identified fatty acid ethyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18;2ω6; (6) C16:0; (7) C18:1ω9. For chromatographic condition see Fig. 2.

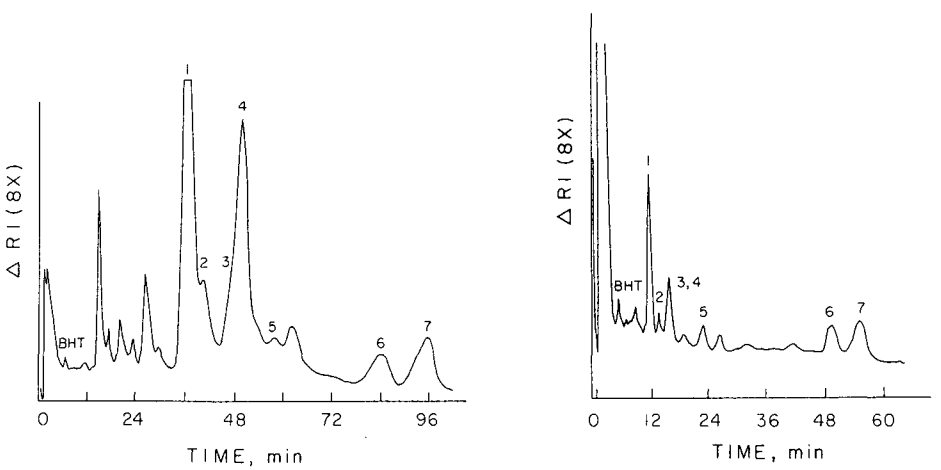

Fig. 7. RP-HPLC of *n*-butyl esters of long-chain fatty acids, derived from sardine oil. Identified fatty acid *n*-butyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18:2ω6; (6) C16-0; (7) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 8. RP-HPLC of benzyl esters of long-chain fatty acids, derived from sardine oil. Identified benzyl esters in order of their elution: (1) C20:5ω3; (2) C14:0; (3) C16:1ω7; (4) C22:6ω3; (5) C18:2ω6; (6) C16:0; (7) C18:1ω9. For chromatographic conditions see Fig. 2.

Fig. 7 *n*-butyl esters; and Fig. 8, benzyl esters. The deterioration in peak shape with increased retention may be due to the decreased solubility of the esters in the mobile phase as well as to the band broadening which occurs with increased retention time.

Peak identification

The peak identification for the methyl and ethyl esters was based on comparison with chromatograms of commercially available standard compounds. In addition, the identification of the methyl ester of EPA was confirmed by NMR and GC–MS. The peaks in the *n*-butyl and benzyl ester chromatograms were assigned by analogy to the methyl and ethyl ester profiles, as standards of these compounds are not readily available. The presence of the ester functionality of benzyl myristate was confirmed by IR.

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THERMODYNAMIC APPROACH TO THE PRACTICAL CHARACTERIZATION OF SOLVENT STRENGTH AND SELECTIVITY OF COMMONLY USED STATIONARY PHASES IN GAS CHROMATOGRAPHY

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SUMMARY

A new approach is proposed for the determination of solvent strength and solvent selectivity in gas chromatography based on thermodynamic considerations. The solvent strength is defined as the partial molar Gibbs free energy of solution for a methylene group per unit solvent volume. Solvent selectivity is determined as the partial molal Gibbs free energy of solution for the test solutes *n*-butylbenzene, 1-octanol, benzodioxan and nitrobenzene. Solvent strength and selectivity values are provided for 24 commonly used liquid phases spanning a wide range of solvent properties.

INTRODUCTION

Chromatographers are frequently faced with the problem of having to select a stationary phase to perform a given separation. In some cases an earlier report in the literature indicating the separation of the same or of a similar sample will provide a solution. In many cases, however, it is more likely that an empirical choice will have to be made or the recommended phase from the literature source is unavailable. If the composition of the sample is known then a stationary phase with complementary selectivity would be a reasonable first choice and if the solvent selectivity of all of the most frequently encountered phases was known then a better selection could be made and redundant phases with similar properties identified for replacement. Stocking many phases with similar properties in the laboratory could be avoided and when a phase not on hand was required for a particular analysis, perhaps following a suggestion from a literature source, the nearest chemical equivalent phase could be substituted with reasonable expectations of obtaining a similar separation. To achieve this goal we need a reasonable model of the way solutes of different kinds interact with common stationary phases in such a way that these phases can be ranked in a quantitative manner by their capacity to enter into specific intermolecular interactions. Unfortunately fundamental approaches have not advanced to the point where an exact model can be put forward to describe the principal intermolecular forces between complex molecules. Chromatographers have come to rely, therefore, on empirical models to estimate the solvent strength and selectivity of stationary

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phases of which the Rohrschneider/McReynolds system of phase constants[1–3], solubility parameters[4,5], the solvent selectivity triangle of Snyder[6,7], Hawkes polarity indices[8], selectivity indices of Evans and Haken[9] and various thermodynamic approaches[10,11] have been most widely used. For a comprehensive review and bibliography see ref. 12. It would be no disrespect to the many workers in this field to state that the system of phase constants promolgated by Rohrschneider/McReynolds is by far the most widely used in practice. McReynolds determined phase constants for over 200 liquid phases and most new phases introduced since this compilation have been characterized using the same protocol. These same phase constants are commonly reported in the catalogs of companies manufacturing or selling stationary phases and few modern text books on chromatography omit a discussion of the use of McReynolds phase constants as an aid to stationary phase selection.

Evidence has come to light in recent years which suggests that the Rohrschneider/McReynolds phase constants may be unreliable for a combination of experimental and theoretical reasons[12–19]. From an experimental point of view the poor retention of some test solutes at the recommended measuring temperature and the failure to account for interfacial adsorption as a significant retention mechanism are the major problems. In fact on many common polar phases the *n*-alkane retention index standards are retained almost exclusively by interfacial adsorption while the test solutes are retained largely by partitioning or by a mixed retention mechanism. Under these circumstances the phase constants derived from retention index differences will be meaningless and subject to substantial changes for different column packings and column types. This observation applies to other methods of determining stationary phase selectivity such as those of Snyder[6], Evans and Haken[9] and Golovnya and Misharina[10] which are based on the use of the retention index system. Theoretical objections are based on the proof that the phase constants are composite terms determined by the solubility of the *n*-alkane retention index standards in the compared phases as well as the magnitude of the selective interactions of the test solutes. In fact, in the majority of cases, the magnitude of the phase constants is determined almost entirely by the properties of the *n*-alkane retention index standards and a high level of correlation exists between the phase constants and various parameters describing the solubility of the *n*-alkanes in the stationary phase. This also explains why the McReynolds phase constants tend to increase monotonously with polarity rather than showing greater variation as predicted by chemical intuition and the results from analyzing test mixtures on different phases.

The keen need for some quantitative scale of stationary phase strength and selectivity has led us to seek a solution which is fundamentally sound and not based on the use of the retention index system. Following the considerations of Golovnya and Misharina[10] we will define the solvent strength of a stationary phase as its capacity for various intermolecular interactions, and solvent selectivity, as the relative capacity of compared solvents for a particular intermolecular interaction. The magnitude of these interactions will be determined as the partial molal Gibbs free energy of solution for a series of test solutes selected to express specific solute–solvent interactions. This paper deals with the establishment of the experimental protocol for measuring the necessary chromatographic parameters and the uncertainty associated with their determination, the selection of the solute test substances for solvent selectivity determination and the cataloging of solvent strength and selectivity values for 24

commonly used stationary phases chosen to encompass the full range of solvent strength values.

EXPERIMENTAL

Unless otherwise stated, all chemicals and solvents were general laboratory or analytical grade in the highest purity available. The silicone polymers SE-30 [poly(dimethylsiloxane)], OV-3 [poly(dimethylmethylphenylsiloxane) containing 10 mole % of phenyl groups], OV-7 [poly(dimethylmethylphenylsiloxane) containing 20 mole % of phenyl groups], OV-11 [poly(dimethylmethylphenylsiloxane) containing 35 mole % of phenyl groups], OV-17 [poly(methylphenylsiloxane)], OV-22 [poly-(phenylmethyldiphenylsiloxane) containing 65 mole % of phenyl groups], OV-25 [poly(phenylmethyldiphenylsiloxane) containing 75 mole % of phenyl groups], OV-105 [poly(cyanopropylmethyldimethylsiloxane)], OV-225 [poly(cyanopropyl-methylphenylmethylsiloxane)], OV-275 [poly(dicyanoallylsiloxane)] and OV-330 [a poly(dimethylsiloxane)–Carbowax copolymer] were obtained from Ohio Valley Specialty Chemical (Marietta, OH, U.S.A.). Squalane (2,6,10,15,19,23-hexamethyl-tetracosane), QF-1 [poly(trifluoropropylmethylsiloxane)], Carbowax 20M [poly-(ethylene glycol)], DEGS [poly(diethylene glycol succinate)], TCEP [1,2,3-tris(2-cyanoethoxy)propane], PPE-5 [poly(phenyl ether) with five rings], column conditioner (a mixture of silanization reagents, No. A7682) and Chromosorb W AW (40–60 or 60–80 mesh) were obtained from Anspec (Ann Arbor, MI, U.S.A.). Tributyl-ammonium 4-toluenesulfonate (TBA pTS), tetrabutylammonium 4-toluenesulfonate (QBA pTS), tetrabutylammonium N-(2-acetamido)-2-aminoethanesulfonate (QBA ACES) and tetrabutylammonium 3-[tris(hydroxymethyl)methylamino]-2-hydroxy-1-propanesulfonate (QBA TAPSO) were prepared as described previously[16,19]. Tetrabutylammonium methanesulfonate (QBA MES) was obtained from Fluka (Ronkonkoma, NY, U.S.A.), tetrabutylammonium picrate (QBA PIC) from RSA Corporation (Ardsley, NY, U.S.A.) and tetraethylammonium 4-toluenesulfonate (QEA pTS) from Aldrich (Milwaukee, WI, U.S.A.).

Column packings containing from 7 to 17% (w/w) of liquid phase were prepared using the rotary evaporator technique[20]. After coating, the damp packings were dried in a fluidized-bed drier and packed into glass columns 1.0–3.0 m × 2 mm I.D. with the aid of suction and gentle vibration. Each column was conditioned overnight at 140°C (120°C for squalane). Column packings prepared with squalane, OV-3, OV-7, OV-11, OV-17, OV-22, OV-25, OV-105, OV-225, OV-330, PPE-5, QF-1 and SE-30 were extensively on-column silanized using repeated injections of 50 μl of column conditioner at 140–150°C (120°C for squalane). Conditioning was considered complete when symmetrical peak shapes and invariant retention times were obtained for dipolar and hydrogen bond donor–acceptor test solutes.

To determine accurate phase loadings the amount of liquid phase coated on the support was determined by Soxhlet extraction[18,21]. The column packing, 0.50–1.50 g $\pm$ 0.2 mg, was placed in a sintered-glass crucible previously dried to constant weight. The crucible was placed in a standard Soxhlet extractor supported by glass beads and covered with a piece of cellulose paper to prevent splashing. The Soxhlet extractor was set to cycle at 4 to 6 times per hour for 72 h using the same solvent as used for coating.

Density as a function of temperature for the liquid phases was determined over

the temperature range 60–130°C using a modified Lipkin bicapillary pycnometer described in ref. 22. The data were fitted to the general equation

$$\rho_t = A - B(t) \quad (1)$$

where ρ_t is the liquid density at temperature t(°C) and A and B are regression coefficients summarized in Table I. Due to its high viscosity, the density of SE-30 was determined with a Gay-Lussac type pycnometer (Ace Glass, Vineland, NJ, U.S.A.). The pycnometer was thermostatted in a large volume oil-bath maintained at 121.2 ± 0.02°C. The experimental density was 0.8007 g ml^{-1}.

For column evaluation a 3700 gas chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.) with heated on-column injectors and a flame ionization detector was used. The column oven temperature was measured with a National Bureau of Standards (NBS)-certified mercury thermometer to ±0.2°C. The column pressure drop was measured with a mercury manometer to ±1.0 mmHg. The carrier gas, nitrogen, was adjusted to a known flow-rate of approximately 20 ml/min using a thermostatted soap-film bubble meter. Samples of 1–100 µl of headspace vapors (usually < 10 µl) were injected onto the column by a gas-tight syringe to approximate the conditions for infinite dilution/zero coverage. Retention data were automatically

TABLE I

COEFFICIENTS FOR DENSITY AS A FUNCTION OF TEMPERATURE (EQN. 1)

Stationary phase	*Coefficients*	
	A	10^4 *B*
Squalane	0.8228	6.014
OV-3	1.0444	13.899
OV-7	1.0713	11.963
OV-11	1.1362	11.771
OV-17	1.1312	9.514
OV-22	1.1741	10.832
OV-25	1.2305	11.693
OV-105	1.0209	11.517
OV-225	1.1098	7.513
OV-275	1.1723	6.539
OV-330	1.1605	11.043
QF-1	1.3025	11.337
Carbowax 20M	1.1490	9.494
DEGS	1.3009	9.969
TCEP	1.1437	9.654
PPE-5	1.2212	8.553
QEA pTS	1.1665	7.756
TBA pTS	1.1024	8.320
QBA pTS	1.0898	7.397
QBA PIC	1.1608	6.854
QBA MES	1.0726	7.509
QBA ACES	1.1275	8.741
QBA TAPSO	1.1552	8.654

recorded using a SP4100 pogrammable computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

The retention of test solutes was determined as the net retention volume given by eqn. 2

$$V_N = \frac{3}{2}(t_R - t_M) F_0 \left(\frac{T_c}{T_a}\right)\left(1 - \frac{P_w}{P_a}\right)\left(\frac{P^2 - 1}{P^3 - 1}\right) \quad (2)$$

where V_N is the net retention volume, t_R the solute retention time, t_M the column holdup time (assumed equal to the retention time of methane at T_c), F_0 the carrier gas flow-rate at the column outlet, T_c the column temperature (K), T_a the ambient temperature (K), P_w the vapor pressure of water at T_a, P_a the ambient pressure, P the column pressure drop equal to P_i/P_a and P_i the column inlet pressure. The gas–liquid partition coefficients were calculated by linear extrapolation to infinite phase volume of plots of V_N^*/V_L *vs.* $1/V_L$ based on eqn. 3[7,13,18]

$$\frac{V_N^*}{V_L} = K_L + (A_{GL}K_{GL} + A_{LS}K_{GLS}) \cdot \frac{1}{V_L} \quad (3)$$

where V_N^* is the net retention volume per gram of packing, V_L the volume of liquid phase per gram of packing, K_L the gas–liquid partition coefficient, A_{GL} the gas–liquid interfacial area, K_{GL} the coefficient for adsorption at the gas–liquid interface, A_{LS} the gas–support interfacial area and K_{GLS} the coefficient for adsorption at the support surface. Values for the partition coefficients of test solutes and the uncertainty in their determination are given in Table II.

The partition coefficients for the homologous series of *n*-alkanes and 2-alkanones were fitted to eqn. 4

$$\log K_L = C(n) + D \quad (4)$$

where C and D are coefficients obtained by linear regression and n the number of carbon atoms for the *n*-alkanes and the number of carbon atoms minus 2 for the 2-alkanones. The coefficients obtained by linear regression are given in Table III.

The partial molar Gibbs free energy of solution, ΔG_k^0, for the test solutes was calculated from the gas–liquid partition coefficient, K_L^X, according to eqn. 5

$$(\Delta G_k^0 X)^P = -2.3RT_c \log K_L^X \quad (5)$$

where $(\Delta G_k^0 X)^P$ is the partial molar Gibbs free energy of solution of solute X on phase P and R is the universal gas constant (1.987 cal mol^{-1} K^{-1}). Likewise, the partial molal Gibbs free energy of solution was defined by eqn. 6

$$(\Delta G_m^0 X)^P = -2.3RT_c \log (10^3 K_L^X / RT_c\rho_c) \quad (6)$$

where $(\Delta G_m^0 X)^P$ is the partial molal Gibbs free energy of solution for solute X on phase P and ρ_c the density of the stationary phase at the column temperature.

TABLE II

GAS–LIQUID PARTITION COEFFICIENTS FOR TEST SOLUTES AT 121.4°C (STANDARD DEVIATION IS GIVEN IN PARENTHESIS)

Stationary phase	*Test solute*				
	Benzene	*n-Butylbenzene*	*cis-Hydrindane*	*1-Octyne*	*1-Dodecyne*
Squalane	32.1 (0.9)	437.1 (7.2)	348.1 (4.9)	114.8 (1.7)	1124.1 (17.9)
SE-30	19.7 (1.3)	187.3 (9.6)	144.0 (9.3)	62.1 (2.9)	458.3 (23.7)
OV-3	24.6 (0.8)	238.1 (4.0)	169.1 (4.3)	75.1 (2.1)	563.6 (10.9)
OV-7	26.4 (0.6)	265.4 (7.5)	179.9 (5.4)	80.9 (2.4)	603.2 (16.9)
OV-11	27.4 (1.4)	284.5 (8.7)	181.2 (6.9)	82.0 (3.7)	600.2 (17.7)
OV-17	24.7 (1.1)	282.8 (5.4)	173.3 (3.3)	79.7 (1.4)	563.2 (14.4)
OV-22	27.3 (1.1)	261.5 (3.6)	157.6 (1.6)	73.7 (2.2)	454.4 (6.2)
OV-25	31.1 (1.2)	265.9 (2.2)	152.5 (0.8)	73.8 (1.7)	474.5 (8.5)
OV-105	22.8 (0.5)	212.8 (6.2)	147.3 (5.0)	67.8 (1.9)	504.1 (14.5)
OV-225	24.8 (0.8)	194.0 (5.4)	86.4 (1.4)	44.6 (0.1)	300.2 (6.5)
OV-275	13.3 (1.5)	45.3 (2.8)	17.4 (1.8)	10.2 (1.8)	19.3 (1.7)
OV-330	32.5 (1.6)	273.9 (2.3)	127.7 (2.1)	74.8 (1.9)	496.7 (5.0)
QF-1	16.4 (1.1)	112.5 (2.1)	65.8 (0.8)	28.8 (1.4)	186.0 (3.1)
Carbowax 20M	33.6 (2.3)	222.0 (1.2)	85.6 (2.0)	53.2 (2.0)	355.1 (3.1)
DEGS	21.8 (0.6)	105.3 (3.4)	33.3 (2.2)	24.3 (1.6)	122.1 (8.0)
TCEP	25.4 (0.9)	98.6 (1.7)	27.0 (1.5)	21.9 (1.1)	73.4 (1.4)
PPE-5	39.3 (0.8)	423.2 (6.6)	220.4 (3.0)	98.6 (1.2)	738.8 (11.0)
QEA pTS	18.3 (0.7)	68.9 (1.5)	16.1 (0.4)	11.3 (0.8)	62.5 (1.9)
TBA pTS	29.3 (1.1)	191.3 (3.2)	74.8 (0.9)	36.5 (2.3)	308.2 (5.9)
QBA pTS	30.6 (0.7)	187.9 (4.2)	75.5 (2.1)	38.6 (1.1)	317.8 (12.2)
QBA PIC	35.0 (3.5)	240.6 (1.9)	89.8 (2.1)	38.5 (3.7)	316.2 (5.5)
QBA MES	28.8 (2.1)	178.7 (5.5)	70.4 (3.6)	35.1 (3.4)	320.0 (13.4)
QBA ACES	20.6 (0.9)	84.8 (1.8)	31.2 (1.7)	16.5 (1.0)	94.9 (5.2)
QBA TAPSO	17.3 (1.4)	59.9 (2.5)	23.8 (0.9)	10.7 (0.8)	52.7 (1.0)

	1-Butanol	*2-Methyl-2-pentanol*	*Dodecafluoroheptanol*	*1-Octanol*	*Phenol*
Squalane	18.1 (0.9)	39.1 (0.6)	33.7 (0.4)	322.4 (5.5)	111.3 (3.7)
SE-30	17.3 (0.9)	26.6 (1.3)	32.9 (1.6)	191.2 (6.5)	102.5 (3.8)
OV-3	22.5 (1.8)	32.6 (0.9)	41.9 (0.8)	246.3 (7.9)	148.0 (3.5)
OV-7	23.8 (0.9)	34.3 (0.8)	44.3 (1.3)	272.7 (9.5)	182.5 (7.1)
OV-11	24.1 (1.6)	33.6 (1.3)	42.4 (1.4)	288.1 (7.0)	217.0 (6.7)
OV-17	24.4 (2.0)	32.1 (1.3)	36.5 (1.2)	284.5 (5.6)	218.4 (9.1)
OV-22	22.2 (1.1)	31.0 (1.2)	36.2 (1.8)	246.5 (9.4)	239.2 (13.5)
OV-25	29.6 (2.1)	27.1 (0.4)	28.4 (1.2)	233.0 (3.0)	253.1 (3.8)
OV-105	23.3 (0.8)	33.6 (0.5)	64.3 (2.6)	248.4 (6.6)	208.3 (4.3)
OV-225	35.3 (0.6)	37.6 (1.0)	131.4 (1.9)	353.3 (7.7)	1033.0 (19.7)
OV-275	34.3 (1.0)	17.7 (1.2)	65.1 (8.2)	119.5 (5.9)	2068.5 (35.8)
OV-330	62.2 (0.8)	57.7 (0.5)	334.5 (12.6)	614.2 (12.1)	
QF-1	19.3 (0.9)	27.6 (0.6)	71.7 (0.6)	148.7 (2.5)	104.2 (0.7)
Carbowax 20M	73.8 (1.2)	56.7 (1.0)	287.7 (3.8)	638.1 (6.2)	5267.0 (77.1)
DEGS	51.2 (1.4)	39.8 (0.9)	168.7 (6.7)	305.5 (8.6)	2718.9 (93.9)
TCEP	60.7 (0.6)	40.4 (0.9)	166.3 (4.0)	290.2 (1.9)	3281.8 (9.8)
PPE-5	35.0 (0.5)	49.6 (1.1)	50.0 (1.0)	476.5 (8.6)	514.3 (8.1)
QEA pTS	134.8 (2.2)	58.9 (2.1)	1618.2 (28.4)	672.3 (10.0)	–
TBA pTS	166.3 (4.4)	114.8 (2.6)	2221.9 (33.8)	1441.9 (5.7)	–
QBA pTS	290.2 (5.6)	166.8 (3.7)	5911.5 (109.0)	2494.7 (45.0)	–
QBA PIC	68.2 (2.6)	57.7 (3.0)	263.7 (4.2)	627.1 (8.4)	–
QBA MES	325.3 (9.7)	181.9 (7.2)	–	2744.1 (99.6)	–
QBA ACES	207.1 (3.4)	94.0 (3.0)	–	1217.5 (36.0)	–
QBA TAPSO	106.6 (3.0)	57.3 (2.5)	–	500.5 (1.7)	–

TABLE II *(continued)*

Stationary phase	*Test solute*				
	2,4,6-Trimethyl-phenol	*Benzonitrile*	*1-Nitropropane*	*1-Nitropentane*	*Nitrobenzene*
Squalane	806.9 (17.5)	168.5 (2.9)	31.3 (0.7)	123.4 (1.8)	359.7 (5.9)
SE-30	385.5 (18.8)	111.9 (5.1)	25.7 (1.7)	84.5 (4.1)	201.8 (8.9)
OV-3	569.8 (13.0)	177.0 (4.4)	38.3 (1.3)	125.3 (2.6)	320.9 (7.7)
OV-7	711.1 (19.5)	231.0 (6.4)	46.2 (1.3)	155.0 (4.5)	422.6 (12.2)
OV-11	855.5 (24.5)	291.0 (8.6)	54.6 (2.2)	179.4 (6.1)	535.4 (15.3)
OV-17	916.1 (17.1)	319.9 (6.6)	57.3 (0.9)	186.9 (3.8)	594.3 (11.1)
OV-22	922.8 (12.7)	334.7 (5.1)	57.9 (1.6)	178.8 (2.7)	611.7 (8.5)
OV-25	949.0 (17.3)	345.1 (6.7)	57.8 (3.1)	176.3 (3.5)	631.9 (9.9)
OV-105	601.5 (16.2)	170.3 (5.4)	39.6 (1.1)	129.1 (4.4)	300.0 (8.8)
OV-225	1708.6 (36.4)	512.8 (9.4)	99.4 (1.9)	298.9 (5.7)	892.1 (9.1)
OV-275	1337.2 (43.9)	445.3 (9.5)	96.1 (1.5)	179.3 (8.5)	704.7 (18.2)
OV-330	–	626.1 (3.3)	99.8 (0.7)	290.9 (2.3)	1128.0 (6.0)
QF-1	312.0 (2.0)	274.5 (2.7)	76.4 (1.2)	213.9 (3.5)	440.3 (4.6)
Carbowax 20M	5246.4 (67.5)	878.6 (40.0)	122.2 (1.9)	315.3 (1.7)	1541.5 (32.9)
DEGS	2778.0 (92.2)	614.5 (21.5)	100.6 (3.4)	225.8 (8.2)	1109.0 (41.5)
TCEP	2933.2 (15.6)	810.4 (4.5)	156.5 (2.6)	305.3 (4.7)	1338.2 (5.2)
PPE-5	2080.9 (35.5)	602.8 (9.5)	87.3 (0.8)	302.9 (3.7)	1291.0 (21.7)
QEA pTS	–	766.1 (9.7)	112.0 (2.1)	220.2 (2.5)	1223.2 (17.2)
TBA pTS	–	979.8 (17.4)	154.8 (3.8)	414.9 (2.8)	1732.2 (26.1)
QBA pTS	–	1275.7 (21.7)	194.7 (2.9)	502.6 (9.3)	2246.3 (39.6)
QBA PIC	–	1078.9 (9.2)	166.0 (0.6)	478.5 (3.7)	1910.7 (19.7)
QBA MES	–	1204.1 (40.6)	184.9 (5.6)	479.7 (15.3)	2133.9 (70.3)
QBA ACES	–	898.2 (12.1)	133.1 (1.0)	281.8 (5.9)	1515.2 (9.0)
QBA TAPSO	–	613.1 (4.5)	95.4 (1.2)	189.9 (4.6)	1017.4 (1.5)
	1,1,2,2-Tetra-chloroethane	*Pyridine*	*2,4,6-Trimethyl-pyridine*	*Aniline*	*N-Methylaniline*
Squalane	141.7 (2.0)	43.6 (1.2)	256.6 (5.0)	170.3 (4.4)	350.5 (7.4)
SE-30	75.5 (3.7)	31.3 (2.1)	122.4 (7.3)	109.5 (5.2)	178.9 (7.6)
OV-3	108.9 (2.9)	42.0 (1.8)	163.3 (4.0)	162.6 (3.2)	267.8 (5.8)
OV-7	136.1 (4.8)	50.5 (2.0)	192.0 (4.3)	207.5 (5.0)	342.4 (7.4)
OV-11	155.6 (5.2)	57.3 (3.3)	218.9 (7.0)	259.5 (6.6)	426.5 (12.0)
OV-17	163.3 (4.7)	60.8 (2.7)	228.2 (5.0)	288.9 (4.9)	471.0 (9.9)
OV-22	161.3 (2.1)	67.5 (1.1)	225.9 (4.2)	321.3 (5.7)	506.1 (4.1)
OV-25	165.7 (1.4)	68.1 (4.9)	234.0 (5.8)	348.4 (2.7)	585.9 (10.5)
OV-105	104.7 (3.0)	39.0 (1.1)	146.4 (4.8)	163.4 (4.0)	256.7 (7.1)
OV-225	222.7 (5.2)	73.9 (1.2)	194.6 (3.3)	569.0 (12.0)	711.2 (9.7)
OV-275	149.6 (4.0)	70.8 (2.6)	81.3 (3.1)	933.6 (13.6)	708.2 (18.8)
OV-330	–	89.1 (0.7)	270.0 (2.7)	938.2 (12.2)	1058.6 (12.6)
QF-1	65.8 (0.8)	46.1 (1.3)	96.1 (1.0)	146.6 (1.2)	211.5 (2.4)
Carbowax 20M	509.1 (5.4)	111.7 (1.7)	270.6 (1.3)	1687.1 (18.7)	1459.3 (20.1)
DEGS	271.0 (9.1)	134.9 (12.1)	346.6 (28.8)	1235.4 (31.9)	977.9 (37.6)
TCEP	258.5 (0.8)	125.0 (1.2)	197.7 (3.4)	1646.5 (18.3)	1271.4 (11.2)
PPE-5	291.1 (4.5)	95.7 (3.6)	426.9 (6.9)	643.2 (10.0)	1029.5 (18.1)
QEA pTS	384.4 (6.7)	–	–	–	–
TBA pTS	593.0 (14.9)	–	–	–	–
QBA pTS	1006.6 (25.1)	136.0 (3.4)	–	3880.4 (71.1)	3393.9 (36.1)
QBA PIC	328.9 (2.9)	138.3 (4.2)	272.2 (8.9)	1789.4 (6.4)	1872.6 (18.0)
QBA MES	–	128.9 (3.3)	179.5 (7.3)	4120.4 (117.9)	3512.9 (119.0)
QBA ACES	–	111.0 (2.2)	115.9 (1.3)	3112.7 (29.9)	2282.5 (29.3)
QBA TAPSO	–	98.7 (2.1)	106.7 (4.3)	1776.1 (10.1)	1308.8 (3.2)

(Continued on p. 242)

TABLE II *(continued)*

Stationary phase	*Test solute*				
	N,N-Dimethyl-aniline	*2,6-Dimethyl-aniline*	*Dioxane*	*Anisole*	*Dihexyl ether*
Squalane	486.9 (6.7)	697.0 (11.4)	31.4 (0.4)	150.1 (1.9)	1710.1 (27.4)
SE-30	215.2 (11.7)	315.0 (14.2)	23.1 (1.3)	80.8 (4.5)	614.0 (31.7)
OV-3	306.8 (6.3)	480.7 (10.8)	30.7 (1.4)	110.3 (1.9)	746.2 (15.0)
OV-7	376.2 (11.1)	618.6 (17.9)	35.2 (0.9)	129.9 (3.3)	785.6 (21.8)
OV-11	451.8 (13.6)	775.0 (18.8)	39.4 (1.4)	151.1 (4.9)	766.5 (22.1)
OV-17	483.7 (9.6)	857.6 (13.4)	41.9 (1.2)	159.3 (3.3)	704.4 (18.1)
OV-22	550.5 (8.4)	906.9 (11.3)	42.3 (1.1)	165.2 (3.9)	565.9 (12.2)
OV-25	535.0 (7.7)	973.8 (19.8)	42.5 (0.8)	172.2 (3.8)	527.9 (16.1)
OV-105	275.1 (8.2)	452.4 (12.5)	28.7 (0.5)	100.4 (2.9)	659.3 (19.4)
OV-225	485.6 (15.7)	1261.1 (18.4)	45.3 (0.5)	159.7 (3.5)	309.6 (9.3)
OV-275	270.3 (6.5)	1147.8 (31.2)	41.9 (0.8)	98.3 (1.3)	13.9 (2.0)
OV-330	643.7 (5.8)	1826.1 (9.1)	53.3 (1.5)	220.4 (1.3)	451.8 (4.9)
QF-1	214.8 (1.9)	352.7 (3.4)	32.1 (0.8)	78.7 (1.1)	226.2 (1.8)
Carbowax 20M	660.4 (4.7)	2536.5 (29.0)	60.6 (1.8)	248.3 (1.7)	237.0 (0.5)
DEGS	436.8 (13.4)	1816.1 (47.6)	60.6 (3.5)	174.3 (6.4)	57.9 (10.0)
TCEP	505.1 (5.9)	2335.4 (26.9)	77.5 (1.4)	191.1 (2.2)	48.8 (2.8)
PPE-5	943.1 (15.2)	1910.3 (26.7)	69.0 (1.4)	279.0 (5.8)	859.7 (13.1)
QEA pTS	–	–	40.6 (0.6)	148.6 (2.9)	60.9 (8.9)
TBA pTS	–	–	47.8 (1.0)	216.1 (3.9)	225.0 (2.6)
QBA pTS	573.8 (10.5)	3458.4 (64.2)	53.0 (1.0)	231.7 (5.0)	190.0 (4.9)
QBA PIC	873.5 (4.3)	885.8 (13.7)	68.2 (2.6)	258.2 (1.7)	234.7 (2.7)
QBA MES	550.8 (17.3)	4036.6 (128.9)	51.4 (2.0)	221.2 (6.5)	190.4 (13.8)
QBA ACES	364.6 (4.4)	2797.2 (26.9)	44.6 (0.9)	155.3 (1.7)	43.2 (3.1)
QBA TAPSO	294.1 (1.1)	1790.7 (15.8)	38.3 (1.9)	119.8 (2.4)	37.7 (2.3)

	Benzodioxan	*Nonanal*
Squalane	637.6 (11.8)	424.2 (6.6)
SE-30	314.5 (16.6)	228.2 (10.6)
OV-3	493.3 (10.5)	303.8 (6.4)
OV-7	658.0 (18.6)	349.3 (10.9)
OV-11	855.8 (22.3)	374.3 (11.5)
OV-17	967.2 (20.5)	367.9 (7.2)
OV-22	1041.9 (15.4)	324.3(4.5)
OV-25	1130.4 (15.7)	307.4 (6.9)
OV-105	422.1 (12.4)	292.2 (8.8)
OV-225	1022.7 (13.5)	349.5 (5.8)
OV-275	749.2 (21.1)	75.9 (5.9)
OV-330	1645.9 (4.0)	383.4 (3.7)
QF-1	313.7 (3.4)	293.7 (2.2)
Carbowax 20M	2179.7 (30.8)	306.3 (1.8)
DEGS	1597.2 (58.6)	162.9 (5.7)
TCEP	1619.2 (8.3)	173.9 (3.3)
PPE-5	2014.5 (40.7)	581.4 (11.3)
QEA pTS	1364.2 (14.7)	101.9 (2.1)
TBA pTS	1761.8 (30.3)	358.1 (3.9)
QBA pTS	2163.3 (32.4)	377.8 (4.4)
QBA PIC	1814.6 (14.4)	499.1 (5.3)
QBA MES	2036.4 (65.6)	349.7 (12.2)
QBA ACES	1456.0 (16.3)	82.5 (1.8)
QBA TAPSO	994.9 (10.8)	113.1 (5.6)

TABLE III

COEFFICIENTS FOR EQN. 4

Stationary phase	*Homologous series*	*Regression coefficients*			*Range (n)*
		C	*D*	r^2	
Squalane	Alkanes	0.2880	−0.3586	1.00	8–13
	2-Alkanones	0.2940	0.5475	1.00	2–7
SE-30	Alkanes	0.2482	−0.3448	1.00	7–12
	2-Alkanones	0.2567	0.5361	1.00	2–7
OV-3	Alkanes	0.2517	−0.3434	1.00	9–14
	2-Alkanones	0.2542	0.6682	1.00	2–7
OV-7	Alkanes	0.2554	−0.3982	1.00	9–14
	2-Alkanones	0.2589	0.6946	1.00	2–7
OV-11	Alkanes	0.2570	−0.4617	1.00	9–14
	2-Alkanones	0.2635	0.6959	1.00	2–7
OV-17	Alkanes	0.2540	−0.4853	1.00	10–15
	2-Alkanones	0.2610	0.7127	1.00	3–7
OV-22	Alkanes	0.2449	−0.4976	1.00	10–15
	2-Alkanones	0.2541	0.6987	1.00	3–7
OV-25	Alkanes	0.2391	−0.4771	1.00	11–16
	2-Alkanones	0.2391	0.7816	0.99	2–7
OV-105	Alkanes	0.2458	−0.3152	1.00	9–14
	2-Alkanones	0.2556	0.6560	1.00	2–7
OV-225	Alkanes	0.2314	−0.6529	1.00	11—16
	2-Alkanones	0.2318	0.9187	1.00	2–7
OV-275	2-Alkanones	0.1471	0.9170	1.00	3–7
OV-330	Alkanes	0.2378	−0.5334	1.00	10–15
	2-Alkanones	0.2321	0.9301	1.00	2–7
QF-1	Alkanes	0.2070	−0.3535	1.00	11–16
	2-Alkanones	0.2185	0.9768	1.00	2–7
Carbowax 20M	Alkanes	0.2217	−0.6355	1.00	11–16
	2-Alkanones	0.2220	0.9151	1.00	2–7
DEGS	Alkanes	0.1726	−0.9028	0.98	12–16
	2-Alkanones	0.1797	0.9676	1.00	2–7
TCEP	Alkanes	0.1559	−0.7517	0.98	10–16
	2-Alkanones	0.1551	1.2126	1.00	2–7
PPE-5	Alkanes	0.2671	−0.6285	1.00	8–13
	2-Alkanones	0.2702	0.8471	1.00	2–7
QEA pTS	Alkanes	0.1586	−0.9687	0.98	12–16
	2-Alkanones	0.1586	0.8961	1.00	3–7
TBA pTS	Alkanes	0.2172	−0.6023	1.00	11–16
	2-Alkanones	0.2133	1.0266	1.00	3–7
QBA pTS	Alkanes	0.2029	−0.4776	1.00	11–16
	2-Alkanones	0.2092	1.0628	0.99	2–5
QBA PIC	Alkanes	0.2193	−0.6274	1.00	11–16
	2-Alkanones	0.2282	1.1306	1.00	2–7
QBA MES	Alkanes	0.1914	−0.3616	1.00	11–16
	2-Alkanones	0.2250	0.9624	1.00	2–6
QBA ACES	Alkanes	0.1619	−0.7035	0.99	12–16
	2-Alkanones	0.1772	0.9766	1.00	2–7
QBA TAPSO	2-Alkanones	0.1607	0.9408	1.00	2–7

The difference in free energies for solute X on two compared phases is given by eqns. 7 and 8

$$\delta(\Delta G_k^0 X)_{SQ}^{P} = (\Delta G_k^0 X)^P - (\Delta G_k^0 X)^{SQ} \quad (7)$$

$$\delta(\Delta G_m^0 X)_{SQ}^{P} = (\Delta G_m^0 X)^P - (\Delta G_m^0 X)^{SQ} \quad (8)$$

where $\delta(\Delta G_k^0 X)_{SQ}^{P}$ and $\delta(\Delta G_m^0 X)_{SQ}^{P}$ are the difference in the partial molar and molal, respectively, Gibbs free energy of solution for solute X on stationary phase P and squalane used as a non-polar reference phase.

The partial molar Gibbs free energy of solution for a methylene group, $\Delta G_k^0(CH_2)^P$, was calculated according to eqn. 9

$$\Delta G_k^0(CH_2)^P = -2.3RT_c C_p \quad (9)$$

where $\Delta G_k^0(CH_2)^P$ is the partial molar Gibbs free energy of solution for a methylene group on phase P and C_p is the linear regression coefficient from Table III for phase P. This is identical to the partial molal Gibbs free energy of solution for a methylene group. The solvent strength parameter, SSP, is defined by eqn. 10:

$$SSP = \Delta G_k^0(CH_2)^P/\rho_c \quad (10)$$

The solvent strength parameter is formally equivalent to the partial molar Gibbs free energy of solution for a methylene group per unit solvent volume.

RESULTS AND DISCUSSION

The nature of solute–solvent interactions in gas–liquid chromatography are undoubtedly very complex and we are a long way from a fundamental understanding of the forces involved. We must, therefore, characterize these forces in an experimental manner using empirical models which of necessity will be based on a degree of chemical intuition, factual support and common sense. This was the approach taken by Rohrschneider/McReynolds that we now feel must be abandoned for the reasons discussed previously and outlined elsewhere[12–19]. These authors were correct in identifying solvent strength and selectivity as the most useful parameters to characterize the solvent properties of individual liquid phases but, in our opinion, chose the wrong formalism for their calculation. In this paper we will present a new protocol for stationary phase solvent characterization that is based on sound thermodynamic principles and avoids the deficiencies of earlier approaches.

The solvent strength or polarity criteria of a liquid phase is the least satisfactory measure of the properties of a liquid phase. Although most chemists have a reasonable idea of the meaning of polarity and have no difficulty in recognizing water as a polar solvent and hexane as a non-polar one, the use of the term is still subject to considerable misunderstanding. Polarity is frequently used where selectivity is meant, at other times, polarity is taken to be the sum of induction and orientation interactions only. In thermodynamic terms polarity can be defined as the capacity of a solvent for all intermolecular interactions corresponding to the partial molar Gibbs free energy of

solution. In practice, a suitable probe must be selected to determine the polarity of a liquid phase and to enable phases to be ranked in order of polarity. Since polarity is not a unique property of a molecule but a composite expression for several different interactions there is no single substance that can be defined as polar. Several empirical solutions to this problem have been suggested previously and are reviewed elsewhere[12,23]. We will not discuss these further.

The measure of solvent strength cannot be based on the affinity on a polar test solute for a stationary phase as it is impossible to define a test solute expressing the singular character of polarity. The only reasonable approach is to consider the reverse situation, the reluctance of a polar phase to accept a non-polar test solute[10,24–29]. Theoretically perfluoroalkane solvents exhibit the least polar interactions but practically the *n*-alkanes are more useful since hydrocarbon type standards are more readily available.

The partial molar or molal Gibbs free energy of solution for a methylene group is easily determined from the retention characteristics of any suitable homologous series provided a few precautions are observed. The test solutes must be retained exclusively by gas–liquid partitioning or corrected for the contribution from interfacial adsorption. Although chemical intuition would favor the use of the *n*-alkanes as test solutes in practice they are frequently retained largely or exclusively by interfacial adsorption on polar phases[12–19,29]. In a number of cases it is possible to calculate meaningful values for the free energies. This is far less of a problem for test solutes of intermediate polarity such as the 2-alkanones or fatty acid methyl esters. For example, Fig. 1 shows a plot of V_N^*/V_L *vs.* $1/V_L$ for tridecane and 2-octanone on a number of representative phases. The relative contribution of interfacial adsorption to retention is indicated by the slopes of the plots and the contribution of partitioning by the intercept on the V_N^*/V_L axis. Partitioning is the dominant retention mechanism for 2-octanone (and other 2-alkanones) while interfacial adsorption increases in importance with polarity for tridecane. On the most polar phases interfacial adsorption is the dominant or

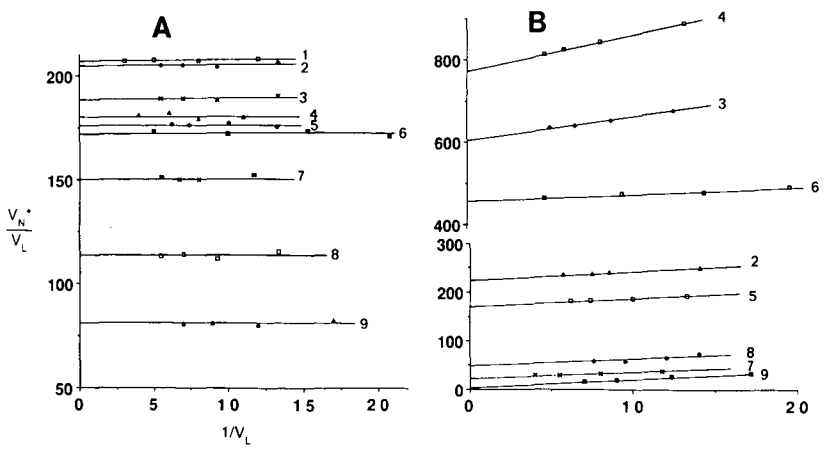

Fig. 1. Plot of V_N^*/V_L *vs.* $1/V_L$ for 2-octanone (A) and tridecane (B) on the stationary phases 1 = squalane, 2 = OV-225, 3 = OV-17, 4 = OV-7, 5 = Carbowax 20M, 6 = OV-22, 7 = TCEP, 8 = DEGS and 9 = OV-275.

exclusive retention mechanism and gas–liquid partition coefficients are very small and cannot be determined with reasonable accuracy. The 2-alkanones are the preferred test solutes for determining $\Delta G_k^0(CH_2)^P$. Provided long chain standards are used the influence of position of the methylene group with respect to the functional group and the difference in free energy of solution for a terminal methyl group compared to a methylene group can be ignored[29,30].

The partial molar Gibbs free energy of solution for a methylene group determined using *n*-alkane and 2-alkanone standards are summarized in Table IV. Where a comparison is possible the agreement is very good with an average difference between scales of 2.9% (S.D. = 3.0%). In one case, QBA MES, a difference of 13.3% was found. The reason for this are not obvious as both sets of data for the *n*-alkanes and 2-alkanones are reproducible in different experiments. Thus, it is reasonable to conclude that the choice of 2-alkanones as reference standards for determining C_p does not produce any significant bias and is preferred to the *n*-alkanes since there are some polar phases for which it is not possible to measure accurate gas–liquid partition

TABLE IV

PARTIAL MOLAR GIBBS FREE ENERGY OF SOLUTION PER METHYLENE GROUP AS A MEASURE OF SOLVENT STRENGTH

Stationary phase	$\Delta G_k^0(CH_2)^P$			*SSP*
	Alkanes	*2-Alkanones*	*Difference*[a] *(%)*	
Squalane	−519	−530	2.1	−728
SE-30	−447	−463	3.5	−578
OV-105	−443	−461	3.9	−523
OV-3	−454	−458	0.8	−523
OV-7	−460	−467	1.5	−504
OV-11	−463	−475	2.5	−478
OV-17	−458	−470	2.6	−463
OV-22	−441	−458	3.7	−439
PPE-5	−481	−487	1.2	−436
OV-225	−417	−418	0	−410
OV-330	−428	−418	−2.4	−407
QBA MES	−345	−398	13.3	−406
OV-25	−431	−431	0	−396
Carbowax 20M	−400	−400	0	−387
TBA pTS	−391	−384	−1.8	−384
QBA PIC	−395	−411	3.9	−381
QBA pTS	−366	−377	2.9	−377
QF-1	−373	−393	5.0	−337
QBA ACES	−292	−319	−8.5	−312
QBA TPSO	–	−290	–	−276
DEGS	−311	−324	4.0	−275
TCEP	−281	−280	0	−273
QEA pTS	−286	−286	0	−267
OV-275	–	−265	–	−243

[a] $\text{Difference} = \dfrac{\Delta G_k^0(CH_2)^{\text{ketone}} - \Delta G_k^0(CH_2)^{\text{alkane}}}{\Delta G_k^0(CH_2)^{\text{ketone}}} \cdot 100.$

coefficients with the *n*-alkane standards. For perspective in comparing the partial molar free energies of solution in Table IV an error of 0.01 in C_p, corresponding to about 3–6% for the data in Table III, would result in a difference of about 18 cal mol^{-1} in the reported values for $\Delta G_k^0(CH_2)^P$. Thus, differences between phases less than about 10 cal mol^{-1} are unlikely to be significant.

Differences in molecular size and uncertainties in the molecular weight of polydisperse phases can influence the free energy largely through the entropy contribution[31,32]. It is necessary to correct for these variations by defining a solvent variable that is independent of solvent molecular weight. The most convenient parameter for this purpose is unit solvent volume. This term is less satisfactory than per unit of mass but is easier to calculate from available data, eqn. 10. This solvent strength parameter, SSP, show a linear increase in polarity with increasing mole percent of phenyl groups ($r = 0.98$) while $\Delta G_k^0(CH_2)^P$ at first shows a decrease in polarity up to 35% phenyl followed by an increase at higher phenyl substitution, Fig. 2. The former behavior seems more reasonable than the latter in terms of expectations from chemical intuition. The high-molecular-weight phase SE-30, a poly(dimethylsiloxane) is ranked as being similar or more polar than several poly(methylphenylsiloxane) phases on the $\Delta G_k^0(CH_2)^P$ scale while it is ranked second to squalane in polarity on the SSP scale and separated from the poly(methylphenylsiloxanes). Similarly, the low-molecular-weight five-ring poly(phenyl ether) PPE-5 is ranked second in polarity to squalane on the $\Delta G_k^0(CH_2)^P$ scale and would be considered less polar than the poly(methylphenylsiloxane) phases with a low incorporation of phenyl groups, again out of keeping with the known general characteristics of these solvents. It is ranked ninth to squalane on the SSP scale flanked by phases that intuition would indicate should have similar properties. The poly(trifluoropropylmethylsiloxane) phase, QF-1, is ranked fifteenth to squalane on the $\Delta G_k^0(CH_2)^P$ scale and seventeenth on the SSP scale. This places it among phases that intuition predicts are much more polar. Perfluorocarbon phases are likely to behave anomalously on this polarity scale due to the unusually weak dispersive interactions of the fluorocarbon portion of the molecule with a methylene group[33,34].

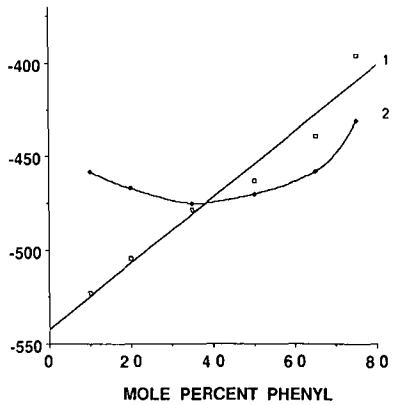

Fig. 2. Plot of the solvent strength parameter, 1, and $\Delta G_k^0(CH_2)^P$, 2, against the mole percent of phenyl groups for a series of poly(methylphenylsiloxane) polymers. Units for *y*-axis are cal · cm^3/mol · g for SSP and cal/mol for $\Delta G_k^0(CH_2)^P$.

In conclusion, the SSP scale seems to be a more appropriate measure of solvent strength than the $\Delta G_k^0(CH_2)^P$ scale. The 2-alkanones are suitable standards for determining the free energy of solution for a methylene group. Fluorocarbon solvents may behave anomalously on this scale. Twenty-four solvents are ranked in increasing polarity using the SSP scale in Table IV.

Whereas to define solvent strength individual solvent variations of a specific nature are ignored so as to rank solvents by a single parameter, to determine solvent selectivity it is these very differences in behavior that we attempt to quantify. There is no doubt that solvent selectivity is more important than solvent strength because it is more clearly related to the ability of individual phases to separate mixtures of similar volatility or similar polarity. To characterize the selectivity of a stationary phase a sufficient number of test solutes are required to adequately characterize the principal intermolecular interactions of dispersion, induction, orientation and donor/acceptor complexation including hydrogen bonding. Unfortunately no solutes interact by a single mechanism except for the limited case of the solution of one alkane in another and in all other cases multiple interactions are involved. The selection of test solutes involves a combination of intuition, chemical information and experiment with the additional constraint that their volatility characteristics must permit the convenient determination of retention time on a wide range of liquid phases. Interpretation will be more straight forward if retention occurs exclusively by gas–liquid partitioning. Rohrschneider chose five substances for this purpose that were later extended by McReynolds to 10 selected from a total of 68 test compounds[2,3,35]. Although the selection of test solutes by McReynolds seems reasonable in terms of sense (chemical intuition) the number of test solutes would seem to be excessive in terms of characterizing stationary phase selectivity. This arises because the data analysis employed by McReynolds was based on determining the number of test solutes required to accurately predict retention indices which can easily exceed the number of probes required to characterize the magnitude of solvent interactions. Applying factor analysis to the data of McReynolds indicates that the precision with which retention indices can be predicted is a stronger function of the number of test solutes employed than their character[36]. Hartkopf *et al.*[37] found that with four test solutes [benzene, nitroethane, *n*-propanol (or chloroform) and dioxane] they could reproduce Rohrschneider's data with similar precision. Lowry *et al.*[38] using a nearest-neighbor pattern recognition technique showed that two sets of three test solutes and several sets of four test solutes gave similar results to those obtained using the first five of the test solutes evaluated by McReynolds. In general agreement with the above studies the McReynolds test solutes benzene, *n*-butanol, 2-pentanone, 1-nitropropane and pyridine (or dioxane) have been most widely used in the contemporary scientific and trade literature for characterizing stationary phase interactions.

In practice the five solutes discussed above are not ideal solutes for investigating chromatographic interactions due to their short retention times on many phases[7,13]. Test solutes that are only weakly retained cannot be expected to adequately characterize stationary phase interactions and are likely to be subject to large experimental errors from small differences in retention time measurements. Some representative data for benzene, butanol, 2-pentanone, nitropropane, pyridine and dioxane on 24 phases spanning a wide polarity range are given in the form of the capacity factor for the solutes on columns containing from 12–15% (w/w) stationary

TABLE V

CAPACITY FACTOR VALUES FOR THE McREYNOLDS TEST SOLUTES AT 121.4°C ON COLUMNS CONTAINING 10–15% (w/w) OF STATIONARY PHASE

Stationary phase	*Test solutes*					
	Benzene	*Butanol*	*2-Pentanone*	*Nitropropane*	*Pyridine*	*Dioxane*
Squalane	1.97	1.25	1.66	1.94	2.72	1.97
SE-30	1.07	0.93	1.07	1.40	1.60	1.27
OV-105	1.09	1.09	1.25	1.94	1.88	1.34
OV-3	1.25	1.19	1.31	1.97	2.13	1.56
OV-7	1.27	1.12	1.36	2.21	2.48	1.67
OV-11	1.12	0.97	1.21	2.18	2.33	1.58
OV-17	0.97	0.85	1.12	2.06	2.12	1.52
OV-22	0.97	0.82	1.00	2.03	2.33	1.52
PPE-5	1.73	1.60	1.93	3.83	4.43	3.00
OV-225	1.06	1.61	1.67	4.24	3.03	1.88
OV-330	1.71	3.03	1.97	5.06	4.50	2.74
QBA MES	1.67	17.36	2.36	10.00	6.91	2.73
OV-25	0.97	0.91	1.00	1.97	2.36	1.52
Carbowax 20M	1.06	2.48	1.21	4.09	3.79	2.03
TBA pTS	1.21	6.97	1.82	6.52	–	2.27
QBA PIC	1.52	3.27	2.82	7.27	6.45	3.27
QBA pTS	1.39	13.13	2.10	8.81	6.13	2.42
QF-1	0.67	0.73	1.60	3.00	1.77	1.27
QBA ACES	0.85	8.27	1.30	5.30	4.48	1.76
QBA TAPSO	0.70	3.94	1.00	3.58	3.73	1.45
DEGS	0.76	1.76	1.06	3.42	4.85	1.97
TCEP	1.14	2.75	2.07	7.04	5.64	3.46
QEA pTS	0.81	6.05	1.03	5.06		1.77
OV-275	0.67	1.36	1.06	3.85	2.82	1.67

phase in Table V. The gas holdup time for these columns is typically about 0.35 min and very few solutes, therefore, have reasonable experimental retention times. For this reason and to permit phase properties to be determined at temperatures greater than 120°C Vernon *et al.* have suggested that *n*-butylbenzene, benzyl alcohol acetophenone, nitrobenzene and aniline[39], or octanol, 2-octanone, 1-nitrohexane and collidine[40] are more suitable test solutes. In an earlier report Schwartz and Mathews[41] used decane, naphthalene, bipyridyl and benzil to evaluate the properties of high melting point phases. A lack of consensus as to the identity and number of test solutes to be used for phase characterization leaves this question open. To define a suitable series of test solutes to characterize stationary phase interactions a number of test solutes discussed above and some additional test solutes were evaluated. Some pertinent physical properties of the selected test solutes are summarized in Table VI[42–44].

The criteria used for selection of appropriate test solutes were that retention of the solute must be dominated by one particular intermolecular interaction, retention should occur as far as possible exclusively by gas–liquid partitioning on all phases, the test solutes must have convenient retention times on all phases (neither too short nor excessively long), test solutes must elute with symmetrical peak shapes on all phases and no two test solutes should have duplicate retention characteristics on all phases. Certain test solutes representing extremes of dipolarity and/or acid–base character-

TABLE VI

PHYSICAL AND SOLVATOCHROMIC PROPERTIES OF POTENTIAL TEST SOLUTES FOR STATIONARY PHASE CHARACTERIZATION

Test solute	*Atmospheric boiling point (°C)*	*Dipole moment (Debyes)*	*Taft constants*[a]		
			π^*	α	β
Benzene	80	0.03–0.1	0.59	0.00	0.10
n-Butylbenzene	183	0.37			
1-Dodecyne	215	0			
1-Butanol	100	1.78	0.47	0.79	0.88
1-Octanol	196	1.72			
Phenol	182	1.50			
2,4,6-Trimethylphenol	220	1.40			
2-Pentanone	101	2.77			
2-Octanone	173	2.46			
Pyridine	115	2.25	0.87	0.00	0.64
2,4,6-Trimethylpyridine	172	2.26			0.78
Aniline	184	1.53			
N-Methylaniline	196	1.68			
N,N-Dimethylaniline	194	1.59	0.90	0.00	
2,6-Dimethylaniline	224	1.63			
Anisole	154	1.25	0.73	0.00	0.22
Dihexyl ether	229	1.18			
Benzodioxan	245	1.43			
Nitropropane	132	3.06			
Nitropentane	180	3.52			
Nitrobenzene	211	3.97	1.01	0.00	0.39
Benzonitrile	188	4.08	0.90	0.00	0.41
1,1,2,2-Tetrachloroethane	147	1.67	0.95		0.00
Dioxane	102	0.40	0.55	0.00	0.55
Dimethyl sulfoxide	189	3.90	1.00	0.00	0.76
Hexamethylphosphoramide	250		0.87	0.00	1.05

[a] Ref. 44.

istics such as dimethyl sulfoxide, hexamethylphosphoramide, tributylphosphine oxide, tripropylamine, 1-octanethiol, dicyclohexylamine, 2-ethylhexanoic acid are unsuitable test solutes due to problems with interfacial adsorption and formation of asymmetric peaks on several phases. In some cases complete adsorption by even the most exhaustively silanized columns occurred in the infinitely dilute solution region.

The test solutes benzene, *n*-butylbenzene, decane and dodecane were evaluated to provide a measure of dispersive interactions. Benzene was known to have insufficient retention for general use but was included as a bench mark for comparison. Fig. 3 shows the changes in $(\Delta G_m^0 X)^P$ for the 24 phases evaluated using the polarity ranking of the phases to assign the arbitrary order used for the *x*-axis of the plot. All four test solutes show the same general characteristics with the exception of a small difference in behavior between the aromatic and alkane solutes on QF-1. The aromatic solutes are retained essentially by partitioning on all phases while dodecane and to a lesser extent dodecyne are retained by a mixed retention mechanism, particularly on the most polar phase. *n*-Butylbenzene has favorable retention characteristics on all phases, Table VII, and was retained as the test solute for dispersive interactions.

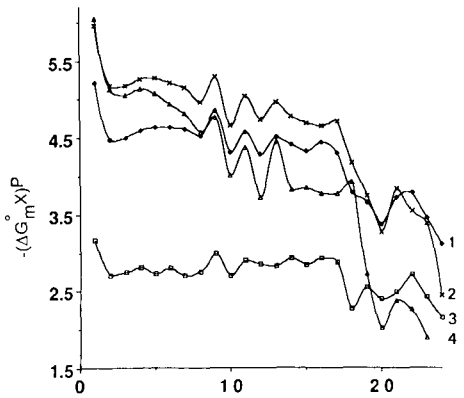

Fig. 3. Plot of $(\Delta G^{0}_{m}X)^{P}$ for X = butylbenzene (1), dodecyne (2), benzene (3) and dodecane (4). Phases are ordered by increasing polarity on the SSP scale (Table IV).

TABLE VII

CAPACITY FACTOR VALUES FOR RECOMMENDED TEST SOLUTES AT 121.4°C ON COLUMNS CONTAINING 10–15% (w/w) OF STATIONARY PHASE

Stationary phase	*Recommended test solutes*			
	Butylbenzene	*Octanol*	*Benzodioxan*	*Nitrobenzene*
Squalane	27.4	20.1	39.9	22.5
SE-30	9.7	9.8	16.2	10.4
OV-105	10.3	12.0	20.5	14.6
OV-3	12.0	12.7	25.0	16.3
OV-7	12.6	13.1	31.4	20.1
OV-11	11.3	11.3	33.6	21.1
OV-17	10.4	10.5	35.2	21.6
OV-22	9.2	9.0	36.7	21.7
PPE-5	18.4	20.8	87.2	56.4
OV-225	7.9	14.4	42.0	36.8
OV-330	14.0	31.0	82.4	56.9
QBA MES	9.6	145.2	109.0	114.2
OV-25	8.9	8.4	27.8	21.4
Carbowax 20M	7.5	21.7	74.6	52.8
TBA pTS	8.5	63.3	76.4	74.9
QBA PIC	10.6	29.9	80.2	83.9
QBA pTS	8.6	112.4	98.0	101.7
QF-1	4.4	5.8	12.4	17.4
QBA ACES	3.4	48.9	58.7	60.6
QBA TAPSO	2.4	20.5	36.8	37.5
DEGS	3.6	10.5	54.3	37.7
TCEP	4.6	13.9	75.9	62.4
QEA pTS	3.1	29.8	61.4	55.3
OV-275	2.1	5.7	30.5	29.4

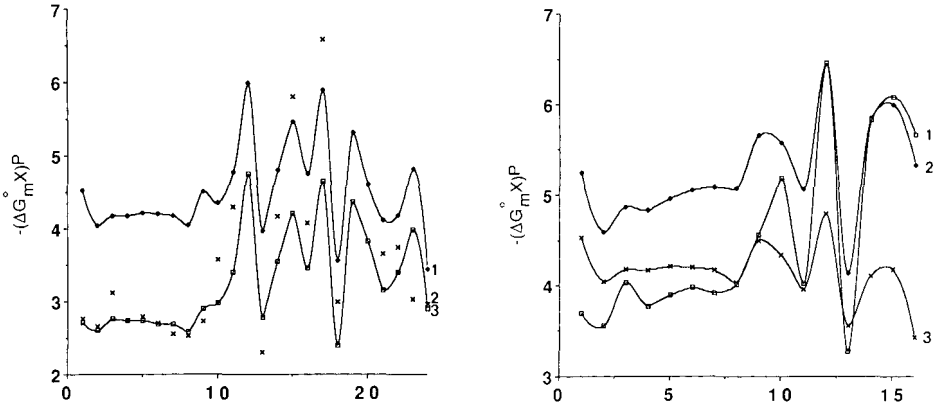

Fig. 4. Plot of $(\Delta G_m^0 X)^P$ for X = octanol (1), 1H,1H,7H-decafluoroheptanol (2) and butanol (3) using the same order of phases given in Fig. 3. 1H,1H,7H-Decafluoroheptanol values are unavailable for some phases so the points are not connected.

Fig. 5. Plot of $(\Delta G_m^0 X)^P$ for X = phenol (1), 2,4,6-trimethylphenol (2) and octanol (3) using the same order of phases given in Fig. 3, skipping OV-330, QBA MES, TBA pTS, QBA pTS, QBA ACES, QBA PIC, QBA TAPSO and QEA pTS.

Alcohols and phenols were selected as test solutes for solvent proton acceptor capacity. The alcohols show both proton donor and acceptor properties with some weak orientation capacity, but in most cases their retention is dominated by solute proton donor interactions. Phenols and 1H,1H,7H-decafluoroheptanol were chosen as additional test solutes to see if more acidic test solutes would show results equivalent to those of the alcohols. They do in the general sense, Figs. 4 and 5, except that poor peak shapes or excessive retention prevented reliable data from being obtained for some phases. This makes them less useful than the alcohols as general test solutes. Octanol is largely retained by gas–liquid partitioning on all phases and has convenient

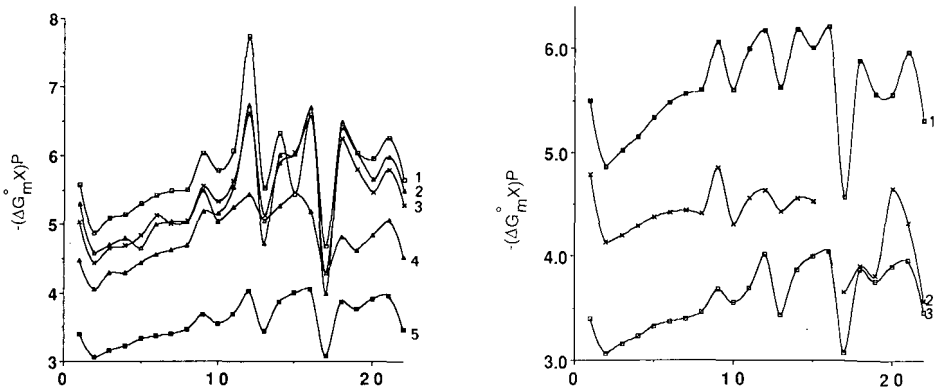

Fig. 6. Plot of $(\Delta G_m^0 X)^P$ for X = 2,6-dimethylaniline (1), aniline (2), N-methylaniline (3), N,N-dimethylaniline (4) and pyridine (5) using the same order of phases given in Fig. 3, skipping TBA pTS and QEA pTS.

Fig. 7. Plot of $(\Delta G_m^0 X)^P$ for X = benzodioxan (1), 2,4,6-trimethylpyridine (2) and pyridine (3) using the same order of phases given in Fig. 3, skipping TBA pTS and QEA pTS.

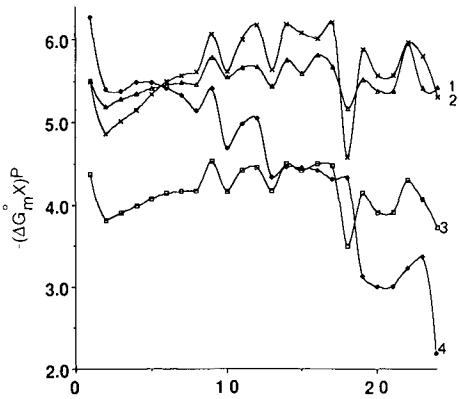

Fig. 8. Plot of $(\Delta G_m^0 X)^P$ for X = dioxane (1), benzodioxane (2), anisole (3) and dihexyl ether (4) using the same order of phases given in Fig. 3.

retention characteristics, Table VII. Octanol was selected as the test solute for solvent proton acceptor capacity.

A number of amines, Figs. 6 and 7, and ethers, Fig. 8, were evaluated as test solutes to characterize solvent proton donor capacity. Ketones and aldehydes were also considered but these solutes behave as if they are retained by a mixed interaction mechanism. Dihexyl ether shows properties more characteristic of dodecane than the other test solutes and is not a suitable probe for solvent proton donor capacity. Aniline and N-methylaniline show substantial proton donor properties and are again unsuitable. Pyridine, 2,4,6-trimethylpyridine and N,N-dimethylaniline show consistent properties but are not eluted with acceptable peak shape from all phases. Benzodioxan, dioxane, anisole and pyridine (on those phases where a comparison is possible) show similar characteristics with benzodioxane having the most favorable retention properties. These test solutes are retained almost exclusively by gas–liquid partitioning on all phases. Benzodioxan was selected as the test solute for solvent proton donor capacity. Its choice is prejudicial on the phases tested showing significant proton donor properties. It cannot be certain that this is the case as there are few common phases in use that are thermally stable and contain a significant percentage of proton donor groups[7]. For the liquid organic salts QBA TAPSO, QBA ACES and TBA pTS, selected because they contain hydroxyl, amide or amine protons in their structure, it was shown that the proton donor capability of these phases is diminished compared to expectations by the involvement of these protons in the formation of intermolecular ion aggregates[4,5]. Thus, one might conclude that none of the phases tested show significant proton donor properties and the selection of a test solute for this interaction should be subject to further review. From a chemical point of view the solutes tested would seem to be reasonable.

Several nitro-containing compounds, benzonitrile and 1,1,2,2-tetrachloroethane were evaluated as test solutes to determine orientation properties, Fig. 9. 1,1,2,2-Tetrachloroethane was found to be unsuitable as it was not eluted from some phases and showed mixed orientation and proton donor characteristics. The nitro-containing compounds and benzonitrile behave in a similar manner indicating that the nitro-containing solutes were not retained by any specific mechanism peculiar to nitro

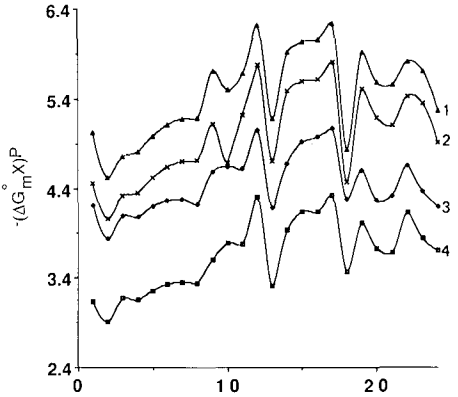

Fig. 9. Plot of $(\Delta G^{0}_{m}X)^{P}$ for X = nitrobenzene (1), benzonitrile (2), nitropentane (3) and nitropropane (4) using the same order of phases given in Fig. 3.

compounds. All solutes were retained almost exclusively by gas–liquid partitioning in the general order 1-nitropentane < benzonitrile < nitrobenzene. Nitrobenzene was arbitrarily selected as the test solute for orientation interactions as either 1-nitropropane or benzonitrile would have been equally acceptable. In terms of retention characteristics 1-nitrohexane might be preferable to 1-nitropropane.

TABLE VIII

SELECTIVITY RANKING OF STATIONARY PHASES BY THEIR ABILITY TO INTERACT WITH *n*-BUTYLBENZENE

Stationary phase	$(\Delta G^{0}_{m}X)^{P}$	$\delta(\Delta G^{0}_{m}X)^{P}_{SQ}$
OV-275	−3.105 ± 0.097	2.085 ± 0.100
QBA TAPSO	−3.356 ± 0.066	1.834 ± 0.070
QEA pTS	−3.449 ± 0.034	1.740 ± 0.043
QBA ACES	−3.650 ± 0.033	1.540 ± 0.042
DEGS	−3.706 ± 0.051	1.483 ± 0.057
TCEP	−3.764 ± 0.027	1.426 ± 0.037
QF-1	−3.768 ± 0.029	1.422 ± 0.039
QBA MES	−4.265 ± 0.048	0.925 ± 0.055
QBA pTS	−4.293 ± 0.028	0.897 ± 0.038
OV-225	−4.300 ± 0.044	0.890 ± 0.051
TBA pTS	−4.302 ± 0.026	0.887 ± 0.037
Carbowax 20M	−4.394 ± 0.009	0.794 ± 0.027
QBA PIC	−4.424 ± 0.012	0.765 ± 0.029
SE-30	−4.460 ± 0.081	0.730 ± 0.085
OV-105	−4.486 ± 0.046	0.704 ± 0.051
OV-25	−4.495 ± 0.013	0.695 ± 0.029
OV-22	−4.515 ± 0.022	0.674 ± 0.034
OV-330	−4.564 ± 0.013	0.626 ± 0.029
OV-3	−4.579 ± 0.026	0.611 ± 0.037
OV-17	−4.597 ± 0.030	0.593 ± 0.040
OV-11	−4.619 ± 0.048	0.570 ± 0.054
OV-7	−4.620 ± 0.044	0.570 ± 0.051
PPE-5	−4.838 ± 0.024	0.361 ± 0.036
Squalane	−5.199 ± 0.026	0

Stationary phase selectivity in order of increasing strength as determined by $(\Delta G_m^0X)^P$ or $\delta(\Delta G_m^0X)_{SQ}^P$ is summarized in Table VIII for *n*-butylbenzene, Table IX for nitrobenzene, Table X for 1-octanol and Table XI for benzodioxan. As would be anticipated retention of *n*-butylbenzene on squalane exceeds that on all other phases. The weakest interactions are shown by DEGS, QF-1, OV-275 and some of the liquid organic salts. The unusual behavior of QF-1 is explained by the low affinity of fluorinated compounds for hydrocarbons as discussed in detail elsewhere[33,34]. There is a poor correlation between SSP and $(\Delta G_m^0X)^P$ and $\delta(\Delta G_m^0X)_{SQ}^P$ where X = *n*-butylbenzene, particularly for the first five phases of minimum polarity (squalane, SE-30, OV-105, OV-3 and OV-7) which are displaced to larger values of SSP compared to the other phases. The correlation coefficient if the five phases of minimum polarity are excluded is $r = 0.94$. The two scales are therefore not redundant as SSP will reflect solvent interactions characteristic of *n*-alkanes and $(\Delta G_m^0X)^P$ characteristic of aromatic hydrocarbons. These differences probably arise from the difference in polarizability and the availability of some weak electron donor capacity for aromatic compounds. Considering the uncertainty in the data for benzene (due to its low retention) there is a reasonable correlation between $(\Delta G_m^0X)^P$ for benzene and *n*-butylbenzene using all phases, $r = 0.88$, and supports the view that the inclusion of benzene into the McReynolds test set was to allow a better estimate of retention indices by accommodating the specific properties of aromatic compounds.

TABLE IX

SELECTIVITY RANKING OF STATIONARY PHASES BY THEIR ABILITY TO INTERACT WITH NITROBENZENE

Stationary phase	$(\Delta G_m^0X)^P$	$\delta(\Delta G_m^0X)_{SQ}^P$
SE-30	−4.519 ± 0.069	0.505 ± 0.074
OV-105	−4.755 ± 0.046	0.269 ± 0.053
OV-3	−4.812 ± 0.038	0.218 ± 0.046
QF-1	−4.833 ± 0.023	0.190 ± 0.034
OV-7	−4.984 ± 0.045	0.039 ± 0.052
Squalane	−5.023 ± 0.026	0
OV-11	−5.114 ± 0.045	−0.091 ± 0.052
OV-25	−5.172 ± 0.025	−0.149 ± 0.036
OV-17	−5.179 ± 0.029	−0.156 ± 0.039
OV-22	−5.181 ± 0.022	−0.158 ± 0.034
OV-275	−5.254 ± 0.041	−0.231 ± 0.048
OV-225	−5.494 ± 0.016	−0.471 ± 0.030
DEGS	−5.549 ± 0.059	−0.526 ± 0.064
QBA TAPSO	−5.573 ± 0.002	−0.554 ± 0.026
OV-330	−5.672 ± 0.008	−0.649 ± 0.027
QEA pTS	−5.701 ± 0.022	−0.678 ± 0.033
PPE-5	−5.711 ± 0.026	−0.688 ± 0.037
TCEP	−5.806 ± 0.006	−0.783 ± 0.026
QBA ACES	−5.907 ± 0.009	−0.884 ± 0.027
Carbowax 20M	−5.912 ± 0.033	−0.888 ± 0.042
TBA pTS	−6.027 ± 0.024	−1.004 ± 0.035
QBA PIC	−6.046 ± 0.016	−1.023 ± 0.030
QBA MES	−6.206 ± 0.052	−1.183 ± 0.058
QBA pTS	−6.232 ± 0.028	−1.209 ± 0.038

TABLE X

SELECTIVITY OF STATIONARY PHASES BY THEIR ABILITY TO INTERACT WITH 1-OCTANOL

Stationary phase	$(\Delta G_m^0 X)^P$	$\delta(\Delta G_m^0 X)_{SQ}^P$
OV-275	−3.865 ± 0.077	1.096 ± 0.082
QF-1	−3.987 ± 0.026	0.974 ± 0.037
OV-25	−4.381 ± 0.000	0.5794 ± 0.027
OV-22	−4.469 ± 0.060	0.492 ± 0.065
SE-30	−4.477 ± 0.053	0.484 ± 0.060
DEGS	−4.540 ± 0.044	0.421 ± 0.052
OV-17	−4.602 ± 0.031	0.359 ± 0.041
OV-3	−4.605 ± 0.050	0.356 ± 0.057
OV-105	−4.607 ± 0.042	0.354 ± 0.049
TCEP	−4.609 ± 0.010	0.352 ± 0.029
OV-11	−4.629 ± 0.038	0.332 ± 0.046
OV-7	−4.641 ± 0.055	0.320 ± 0.061
OV-225	−4.769 ± 0.034	0.192 ± 0.043
PPE-5	−4.931 ± 0.028	0.029 ± 0.039
Squalane	−4.961 ± 0.027	0
QBA TAPSO	−5.0181 ± 0.005	−0.057 ± 0.027
QBA PIC	−5.174 ± 0.021	−0.214 ± 0.034
OV-330	−5.196 ± 0.031	−0.235 ± 0.041
Carbowax 20M	−5.221 ± 0.015	−0.260 ± 0.031
QEA pTS	−5.233 ± 0.023	−0.272 ± 0.035
QBA ACES	−5.735 ± 0.046	−0.775 ± 0.053
TBA pTS	−5.883 ± 0.006	−0.923 ± 0.027
QBA pTS	−6.314 ± 0.028	−1.353 ± 0.039
QBA MES	−6.366 ± 0.130	−1.405 ± 0.133

The strongest orientation interactions are shown by the liquid organic salts with Carbowax 20M, TCEP, OV-330 and DEGS being the most dipolar of the non-ionic phases. The poly(dicyanoallylsiloxane), OV-275, shows only intermediate dipolarity and its status as a polar phase seems to depend largely on its unusually low affinity for alkane and aromatic groups. The least dipolar interactions are shown by the polysiloxane phases lacking a cyanoalkyl group and squalane in keeping with expectations. There is a good correlation between $(\Delta G_m^0 X)^P$ for 1-nitropropane and nitrobenzene, $r = 0.95$. The improvement in the correlation coefficient compared to the data for benzene and *n*-butylbenzene is probably due in large part to the smaller uncertainties in the data for 1-nitropropane.

The strongest proton acceptor interactions are shown by the liquid organic salts as would be anticipated from their published chromatographic applications[45,46]. Carbowax 20M and OV-330 show the strongest proton acceptor interactions of the non-ionic phases. At first sight the position of squalane in Table X might look anomalous and we will return to this point presently. There are a large number of phases just below squalane with similar values for $(\Delta G_m^0 X)^P$ indicating a narrow range of selectivity for a large number of the phases in Table X. Among these phases are OV-275, DEGS, TCEP which show selective orientation and/or weak dispersive-type interactions. Again, given the uncertainties in the data for the McReynolds probe *n*-butanol there is a good correlation with octanol, $r = 0.91$, for the $(\Delta G_m^0 X)^P$ values.

TABLE XI

SELECTIVITY RANKING OF STATIONARY PHASES BY THEIR ABILITY TO INTERACT WITH BENZODIOXAN

Stationary phase	$(\Delta G_m^0 X)^P$	$\delta(\Delta G_m^0 X)_{SQ}^P$
QF-1	−4.571 ± 0.017	0.923 ± 0.033
SE-30	−4.866 ± 0.083	0.629 ± 0.087
OV-105	−5.010 ± 0.070	0.485 ± 0.076
OV-3	−5.149 ± 0.033	0.345 ± 0.044
OV-275	−5.302 ± 0.044	0.192 ± 0.052
OV-7	−5.330 ± 0.044	0.164 ± 0.053
OV-11	−5.481 ± 0.041	0.013 ± 0.050
Squalane	−5.494 ± 0.029	0
QBA TAPSO	−5.556 ± 0.0170	−0.061 ± 0.033
OV-17	−5.560 ± 0.033	−0.065 ± 0.044
OV-22	−5.560 ± 0.023	−0.103 ± 0.037
OV-225	−5.601 ± 0.103	−0.1078 ± 0.107
OV-25	−5.628 ± 0.022	−0.133 ± 0.036
QEA pTS	−5.787 ± 0.017	−0.292 ± 0.033
DEGS	−5.835 ± 0.058	−0.340 ± 0.064
QBA ACES	−5.876 ± 0.018	−0.377 ± 0.033
TCEP	−5.954 ± 0.009	−0.460 ± 0.030
OV-330	−5.968 ± 0.004	−0.473 ± 0.090
QBA PIC	−6.006 ± 0.012	−0.518 ± 0.031
TBA pTS	−6.040 ± 0.027	−0.546 ± 0.039
PPE-5	−6.059 ± 0.032	−0.565 ± 0.042
QBA MES	−6.169 ± 0.051	−0.674 ± 0.058
Carbowax 20M	−6.182 ± 0.022	−0.6877 ± 0.036
QBA pTS	−6.202 ± 0.024	−0.708 ± 0.037

The ranking of phases by their proton donor capacity is rather more compressed than the other scales. The most selective phases are the liquid organic salts, Carbowax 20M, PPE-5 and OV-330. These are also the phases with the highest dipolarity or contain ether linkages. Since none of the most selective phases contain obvious proton donor groups we suspect that the ranking order indicates increasing general solubility of benzodioxan in the stationary phases rather than specific proton donor interactions. TBA pTS, QBA ACES and QBA TAPSO were originally incorporated into this study as examples of proton donor solvents, but as mentioned earlier, the protons available in these solvents are likely involved in anion aggregation making them less available to the solute[16,45]. The need for thermally stable strong proton donor solvents in gas chromatography is probably unfulfilled[7,47].

The Rohrschneider/McReynolds schemes of solvent selectivity incorporated squalane into the protocol as a non-polar reference phase. To determine selectivity a reference phase is in fact unnecessary as it functions only to scale the data and does not affect the magnitude of difference between phases[48]. On theoretical grounds a non-polar reference phase is a reasonable choice as the only solute–solvent interactions involved are dispersion and induction. From a thermodynamic point of view a suitable reference phase would be a chemically defined substance exhibiting minimum selectivity and of sufficiently high molecular weight so as to minimize variations in free energies due to molecular weight differences as discussed by Fritz and Kovats[31]. We have preferred to use the $(\Delta G_m^0 X)^P$ scale in this paper rather than

$\delta(\Delta G_m^0X)_{SQ}^P$ for practical reasons. Of the phases tested squalane is the only one of significant vapor pressure and poor film stability at the meaurement temperature. In terms of selectivity it is the sixth least selective for nitrobenzene, the tenth least selective for 1-octanol and the eight least selective for benzodioxan. It does not meet the thermodynamic criteria for use as a non-selective reference phase. The question is whether this is due to experimental or chemical reasons. The increased retention of 1-octanol and nitrobenzene compared to the other phases of low selectivity might be interpreted as indicating that the silanization procedure used for support deactivation was inadequate. On the other hand, horizontal plots of V_N^*/V_L *vs.* $1/V_L$ were obtained and the uncertainty in the values for the gas–liquid partition coefficients are similar to other phases (reflecting mainly the inaccuracy of determining the phase loading). Note that the gas–liquid partition coefficients are obtained from four independent columns of different phase loading and not just from a single column, packing or experiment. The consistency in the gas–liquid partition coefficients from column to column would only be possible if the contribution from adsorption was the same for all columns and increased in proportion to the phase loading. A set of circumstances that would be very unusual. From Table IV it can be seen that the capacity of squalane for non-polar interactions with a methylene group easily exceeds that of the other phases and the unexpected retention of 1-octanol compared to other weakly selective phases could be due to a greater affinity for the alkane portion of the test solute. This question cannot be resolved at present. In further studies we will investigate the influence of support type and surface area as an additional parameter for determining gas–liquid partition coefficients for squalane and other phases of low selectivity such as Apolane-87 and Apiezon MH which are more stable at the analysis temperature.

The molal standard state was used in calculating the free energies in Tables VIII to XI to avoid complications from differences in the molecular weights of the various phases. In practice the selectivity ranking of the phases is similar if either the molal or molar standard states are used as can be seen from the data in Fig. 10 and Table XII. The two scales are related by a linear equation of the type:

$$(\Delta G_m^0X)^P = E(\Delta G_k^0X)^P + F \tag{11}$$

The slopes, E, vary from 0.91 to 1.07 with correlation coefficients, r, between 0.96 and 1.00. Since most of the slopes deviate from 1.00 the two scales are not identical but the

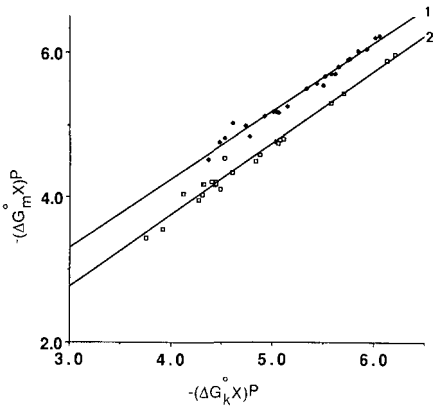

Fig. 10. Plot of $(\Delta G_m^0X)^P$ against $(\Delta G_k^0X)^P$ for X = nitrobenzene (1) and octanol (2).

TABLE XII

CORRELATION OF SELECTIVITY SCALES BASED ON $(\Delta G_m^0X)^P$ AND $(\Delta G_k^0X)^P$ FROM EQN. 11

Test solute	*Linear regression coefficients*			
	E	*F*	*r*	*n*
Butylbenzene	1.0794	0.1322	0.99	24
1-Dodecyne	1.0593	−0.0721	1.00	24
Octanol	0.9900	−0.2048	0.99	24
1H,1H,7H-Dodecafluoroheptanol	0.9832	0.1875	1.00	21
Phenol	0.9504	0.0097	1.00	16
2,4,6-Trimethylphenol	0.9659	0.0806	0.99	14
Aniline	0.9563	0.4113	1.00	22
N-Methylaniline	0.9505	0.4447	0.99	21
N,N-Dimethylaniline	0.9413	0.4592	0.95	22
2,6-Dimethylaniline	0.9330	0.5481	0.98	22
1-Nitropentane	0.9535	0.3676	0.98	22
Nitrobenzene	0.9398	0.4878	0.99	24
Benzonitrile	0.9107	0.6115	0.99	24
2,4,6-Trimethylpyridine	0.9712	0.3088	0.96	21
Anisole	0.9800	0.2492	0.98	22
Benzodioxan	0.9194	0.6273	0.99	23
Dihexyl ether	1.0465	0.0140	1.00	24
Nonanal	1.0375	0.0202	0.98	24

differences are small. In terms of ranking of the phases only those phases with similar values for the free energies would possibly be switched. Given the uncertainties in the values of K_L (Table XI) using the molal or molar standard states does not lead to serious inconsistencies in the determination of solvent selectivity.

In conclusion, two new scales of solvent strength and selectivity have been developed. These scales are based on a different theoretical background than the familiar Rohrschneider/McReynolds approach and lead to different conclusions. In fact the $(\Delta G_m^0X)^P$ scale can be mathematically related to the retention index differences of McReynolds by a complex expression under conditions where retention of the index standards and test solutes occurs exclusively by gas–liquid partitioning[15]. It is shown that any agreement between the two scales is purely coincidental as the values for the retention index differences are conditioned largely by the behavior of the index standards. The two new scales proposed here are largely in keeping with chemical intuition but will be subject to revision as further data is collected and alternative methods of data analysis becomes possible. The raw experimental data which are unbiased by the method of interpretation, we hope will be of value to others in testing solution models of relevance to gas chromatography.

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CHROMSYMP. 1515

CHANGES IN RETENTION AND POLARITY ACCOMPANYING THE REPLACEMENT OF HYDROGEN BY FLUORINE IN TETRAALKYLAMMONIUM ALKYL- AND ARYLSULFONATE SALTS USED AS STATIONARY PHASES IN GAS CHROMATOGRAPHY

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SUMMARY

Fourteen tetraalkylammonium alkanesulfonate, perfluoroalkanesulfonate, and benzene- and pentafluorobenzenesulfonate salts possessing useful chromatographic properties were synthesized. All salts had wide liquid temperature ranges with an upper column operating temperature limit in the range 160–220°C and yielded efficient column packings. The substitution of fluorine for hydrogen in the alkanesulfonate chain resulted in a general reduction in solute non-specific and proton donor interactions accompanied by an increase in solute electron donor interactions. These findings are rationalized in terms of the weak dispersive interactions for solutes with the perfluoroalkane side chains and the ability of fluorine to effect changes in the electron density of the sulfonate group through its inductive effect. On over half the salts studied retention is shown to depend on both gas–liquid partitioning and interfacial adsorption for a wide range of solutes but the relative importance and magnitude of the adsorption contribution to retention is not simply related to the structure of the salts or the incorporation of fluorine into the anion.

INTRODUCTION

The small size and large electronegativity of fluorine, the low degree of polarizability of the carbon–fluorine bond, and the weak intermolecular forces operative in perfluorocarbon solvents are dominant features of the unique chemistry of organofluorine compounds[1]. Fluorocarbons are much more volatile than hydrocarbons of similar molecular weight, they have a higher density and compressibility than hydrocarbons, lower critical temperatures and pressures, and extremely low surface tensions. These unique properties are exploited in numerous industrial processes particularly in the production of synthetic oils and lubricants, plastics and polymers, and pharmaceutical and agricultural chemicals[2].

Numerous attempts have been made to exploit the unique physical and chemical properties of perfluorocarbon solvents in chromatography. Perfluorocarbon stationary phases in gas chromatography (GC) have been used to separate substances of high chemical reactivity such as metal fluorides, halogens, halides of sulfur and phos-

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phorus, etc.[3]. Perfluorocarbon phases show unique selectivity for the separation of fluorocarbon isomers and Freons. However, their use in GC is far from common place since perfluorocarbon phases yield unstable films on diatomaceous supports or glass capillary column walls resulting in columns of low efficiency and low temperature stability. For the above applications it was the unique chemical stability or the selectivity for fluorocarbon isomers that was important and the otherwise poor chromatographic characteristics had to be tolerated. A number of polar group derivatizing reagents for GC contain perfluorocarbon substituents as these reagents generally yield derivatives with retention times less than their hydrocarbon analogues and have a favorable response to the sensitive electron-capture detector. This is probably the most successful application of perfluorocarbon compounds in GC[4,5]. Bonded phase packings containing perfluorocarbon chains have been used in reversed-phase liquid chromatography to selectively separate fluorocarbon compounds and to diminish the retention of solutes compared to their hydrocarbon analogues, a role in which they have only been partially successful, and this combined with poor hydrolytic stability of the packings has diminished interest in their general use[6–10].

Highly-fluorinated polymers have been investigated as a means of extending the molecular weight range of samples that could be analyzed by GC and/or to permit the analysis of thermally labile substances at lower temperatures than is possible with conventional phases. This possibility has been adequately demonstrated using column packings prepared from poly(perfluoroalkyl ethers)[11,12]. However, this approach was limited by two familiar problems associated with perfluorocarbon solvents. Film instability limited the upper column temperature limit to about 250°C and the perfluorocarbon chains provided little masking of support activity which frequently led to poor peak shapes for polar compounds. Taking a different approach we decided to evaluate the use of highly-fluorinated liquid organic salts as stationary phases. The liquid organic salts have good film building properties and are excellent column deactivating agents[13,14]. The alkyl- and arylsulfonate salts have been the most widely studied since these salts frequently have low melting points and wide liquid ranges[15–20]. They also provide a well defined model system to study the influence of the perfluorocarbon group on retention and solvent selectivity in a quantitative manner. Studies of this kind have been lacking in the past[3], and given the problems of coating and immobilizing perfluorocarbon solvents, provide some insight into the properties that could be anticipated for partially fluorinated solvents that would be much easier to synthesize and work with as stationary phases compatible with modern column technology.

EXPERIMENTAL

Tetra-*n*-butylammonium hydroxide [40% (w/w) in water], butanesulfonyl chloride, barium salt of pentafluorobenzenesulfonic acid, benzenesulfonic acid, and the sodium salt of hexanesulfonic acid were obtained from Aldrich (Milwaukee, WI, U.S.A.). Tributylmethylammonium hydroxide [40% (w/w) in water], tetra-*n*-butylammonium methanesulfonate and trifluoromethanesulfonate were obtained from Fluka (Ronkonkoma, NY, U.S.A.), octanesulfonyl chloride from Alfa Products (Danvers, MA, U.S.A.), perfluorooctanesulfonic acid [50% (w/w) solution in water]

from Crescent (Hauppauge, NY, U.S.A.), and perfluorobutanesulfonyl fluoride from SCM (Gainesville, FL, U.S.A.). Chromosorb W-AW (40–60 mesh), HPLC-grade solvents and chemical standards were obtained from Anspec (Ann Arbor, MI, U.S.A.).

Tetra-*n*-butylammonium benzenesulfonate (QBA BS), pentafluorobenzenesulfonate (QBA FBS), butanesulfonate (QBA BuS), octanesulfonate (QBA OS), perfluorooctanesulfonate (QBA FOS), hexanesulfonate (QBA HS), tri-*n*-butylmethylammonium butanesulfonate (TBMA BuS), perfluorobutanesulfonate (TBMA FBuS), octanesulfonate (TBMA OS), perfluorooctanesulfonate, (TBMA FOS), and hexanesulfonate (TBMA HS) were prepared by a neutralization technique. The sulfonyl chlorides or fluorides were first partially hydrolyzed by adding an excess of water which was then removed under vacuum on a rotary evaporator. Sodium salts were converted to the barium salts by precipitation with excess barium hydroxide solution. The barium salts were converted to the acid by addition of an equimolar amount of dilute sulfuric acid followed by removal of barium sulfate by filtration and excess water on a rotary evaporator. For the hydrocarbon salts, the sulfonic acid was added in equimolar amounts to an aqueous solution of the tetraalkylammonium hydroxide. The water was removed on a rotary evaporator and the product dissolved in acetonitrile, treated with decolorizing charcoal (previously cleaned by Soxhlet extraction with methylene chloride), and filtered through a bed of celite. The solvent was removed on a rotary evaporator and the product dried under high vacuum (< 0.2 mm Hg). The perfluorocarbon salts were prepared in a similar manner, however, after the neutralization reaction the product precipitated out of the aqueous solution. It was filtered off, washed three times with cold water, and dried under vacuum (< 0.2 mm Hg). The identity as well as the purity of the salts was confirmed by IR and NMR spectroscopy, Table I. IR spectra were obtained as a smear on a sodium chloride plate using a Nicolet 20DX Fourier transform infrared spectrometer (Madison, WI, U.S.A.) at 1 cm^{-1} resolution. ^{1}H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded on a General Electric QE-300 (Freemont, CA, U.S.A.) spectrometer with the salts dissolved in deuterated chloroform.

Liquid densities as a function of temperature for the tetraalkylammonium salts were determined using a modified Lipkin bicapillary pycnometer[16]. Density relationships were fitted to the equation

$$\rho_t = A - B(T) \tag{1}$$

where ρ_t is the density of the organic salt at temperature T (°C) and A and B are constants determined by linear regression (Table II).

Column packings containing 5–20% (w/w) of salt on Chromosorb W-AW were prepared using the rotary evaporator technique with acetonitrile as the slurry solvent. After coating the packings were dried in a fluidized-bed drier and packed into glass columns (2–3.5 m × 2 mm I.D.) with the aid of suction and gentle vibration. Individual phase loadings were determined by exhaustive Soxhlet extraction, 20–30 h, of the column packing materials with acetonitrile[21].

For column evaluation a Varian 3700 gas chromatograph (Palo Alto, CA, U.S.A.) with heated on-column injectors and a flame-ionization detector was used. The column temperature was stabilized at 121°C (±0.2°C). Nitrogen was used as the

TABLE I

SPECTRAL FEATURES FOR TETRAALKYLAMMONIUM SULFONATE SALTS

Salt	*Principal IR bands (cm^{-1})*	*NMR chemical shifts*	
		^{1}H *Spectra*[a]	^{13}C *Spectra*
QBA BuS	2971, 2884, 1470 1350, 1264, 1211 1131, 1052, 1005 872, 799, 732	cation: 0.94 (12), 1.34 (8) 1.64 (8), 3.13 (8) anion: 0.80 (3), 1.38 (2) 1.73 (2), 2.78 (2)	cation: 58.86, 29.08, 19.75, 13.68 anion: 51.79, 26.96, 22.00, 13.76
QBA FBuS	2964, 2877, 1470 1384, 1198, 1032 886, 746	cation: 0.94 (12), 1.38 (8) 1.58 (8), 3.24 (8)	cation: 58.31, 23.26, 19.24, 12.68
QBA HS	2957, 2931, 2877 1470, 1384, 1191 1038, 885, 746	cation: 1.03 (12), 1.49 (8) 1.66 (8), 3.32 (8) anion: 0.88 (3), 1.29 (4) 1.87 (4), 2.80 (2)	cation: 58.95, 24.18, 19.84 13.77 anion: 52.36, 31.77, 28.91 25.59, 22.59, 14.15
QBA OS	2969, 2866, 1682 1476, 1270, 1160 1098, 884, 742	cation: 1.01 (12), 1.43 (8) 1.64 (8), 3.22 (8) anion: 0.86 (3), 1.34 (10) 1.58 (2), 2.57 (2)	cation: 58.81, 24.02, 19.69 13.61 anion: 61.97, 52.12, 31.77, 29.31, 29.09, 25.15, 22.57
QBA FOS	2964, 2877, 1642 1516, 1483, 1383 1244, 1098, 1044 985, 885, 793	cation: 1.00 (12), 1.45 (8) 1.65 (8), 3.30 (8)	cation: 58.45, 23.39, 19.34 12.73
QBA BS	2964, 2878, 1483 1244, 885, 793 633	cation: 0.98 (12), 1.38 (8) 1.59 (8), 3.24 (8) anion: 7.89 (2), 7.29 (3)	cation: 58.71, 23.95, 19.62, 13.35 anion: 147.63, 128.65, 127.63 126.12
QBA FBS	2964, 2878, 1470 1384, 1350, 1271 1125, 1052, 1005 886, 799, 733	cation: 0.94 (12), 1.39 (8) 1.58 (8), 3.09 (8)	cation: 58.70, 23.00, 19.50 13.50
TBMA BuS	2969, 2879, 1772 1489, 1200, 1059 883, 748	cation: 0.96 (9), 1.34 (6) 1.58 (6), 3.09 (3) 3.30 (6) anion: 0.79 (3), 1.38 (2) 1.73 (2), 2.78 (2)	cation: 58.86, 24.08, 19.75, 13.68 anion: 51.79, 26.96 22.01, 13.76
TBMA FBuS	2964, 2877, 1470 1384, 1198, 1032 886, 746	cation: 0.96 (9), 1.38 (6) 1.58 (6), 3.24 (6) 3.10 (3)	cation: 61.38, 23.65, 19.23, 12.68
TBMA HS	2964, 2931, 2878 1470, 1384, 1191 1038, 892, 779 739, 713	cation: 1.02 (9), 1.43 (6) 1.65 (6), 3.24 (3) 3.37 (6) anion: 0.86 (3), 1.28 (4) 1.81 (2), 2.77 (2)	cation: 61.38, 48.83, 24.42 19.79, 13.77 anion: 52.32, 31.74, 28.84 25.54, 22.59, 14.12

TABLE I (*continued*)

Salt	*Principal IR bands (cm^{-1})*	*NMR chemical shifts*	
		^1H Spectra[a]	*^{13}C Spectra*
TBMA OS	2964, 2932, 2871 1630, 1470, 1377 1191, 1038, 892 779, 739	cation: 1.01 (9), 1.45 (6) 1.65 (6), 3.37 (6) 3.24 (3) anion: 0.83 (3), 1.81 (2) 2.78 (2), 3.37 (2) 1.25 (8)	cation: 61.18, 24.15, 19.56 13.53 anion: 52.09, 48.71, 31.71 29.27, 29.04, 28.96 25.33, 22.49
TBMA FOS	2970, 2885, 1696 1484, 1264, 1211 1151, 1058, 965 912, 766, 746	cation: 1.01 (9), 1.44 (6) 1.60 (6), 3.28 (6) 3.10 (3)	cation: 61.62, 24.31, 19.64 13.53

[a] Number in parentheses denotes the number of protons integrated at the chemical shift.

carrier gas at an accurately known flow-rate of approximately 20 ml/min determined with a thermostated soap-film bubble meter. The column pressure drop was determined with a mercury manometer (± 1 mmHg). Samples were injected as headspace vapors to approximate the infinite dilution condition in the linear portion of the sorption isotherm as indicated by the independence of retention volume on sample size.

The net retention volume was determined using eqn. 2

$$V_N = \frac{3}{2}\frac{(P^2 - 1)}{(P^3 - 1)}(t_R - t_m)F_a\left(\frac{T_c}{T_a}\right)\left(1 - \frac{P_W}{P_a}\right) \quad (2)$$

TABLE II

DENSITY AS A FUNCTION OF TEMPERATURE FOR TETRAALKYLAMMONIUM SULFONATE SALTS

Salt	*Temperature range of measurements (°C)*	*Coefficients*		*S.D. in the intercept*
		A	*10^4B*	
QBA MS	100–134	1.0421	7.321	0.0003
QBA FMS	120–142	1.1332	8.092	0.0006
QBA BuS	70–130	1.0119	7.396	0.0012
QBA FBuS	65–120	1.2487	11.538	0.0054
QBA HS	70–125	1.0586	7.250	0.0013
QBA OS	75–130	1.0537	8.509	0.0014
QBA FOS	85–140	1.4813	13.911	0.0060
QBA BS	105–145	1.1278	9.466	0.0036
QBA FBS	75–120	1.2368	8.914	0.0021
TBMA BuS	95–130	1.0318	7.003	0.0015
TBMA FBuS	95–125	1.4203	18.239	0.0068
TBMA HS	90–125	1.0395	7.690	0.0023
TBMA OS	95–130	1.0270	8.133	0.0024
TBMA FOS	105–130	1.4746	11.461	0.0034

where P is P_i/P_a (P_i being the column inlet pressure and P_a the column outlet pressure), t_R the retention time, t_m the gas hold-up time (assumed to be equal to the retention time of methane at T_c), F_a the column flow-rate measured at P_a and T_a, T_c the column operating temperature (K), T_a the ambient temperature, and P_W the vapor pressure of water at T_a. Gas–liquid partition coefficients were calculated by linear extrapolation of plots of V_N^*/V_L *vs.* $1/V_L$ to infinite phase volume based on eqn. 3[14,20,22,23]

$$\frac{V_N^*}{V_L} = K_L + (A_{LS}K_{LS} + A_{GS}K_{GS})\frac{1}{V_L} \tag{3}$$

where V_N^* is the net retention volume per gram of packing, V_L the volume of liquid phase per gram of packing, K_L the gas–liquid partition coefficient, A_{LS} the surface area of the liquid phase, K_{LS} the coefficient for adsorption at the gas–liquid interface, A_{GS} the available surface area of the support surface, and K_{GS} the coefficient for adsorption at the gas support interface. Values for the gas–liquid partition coefficients of test solutes are given in Table III together with the uncertainty in their determination. The partition coefficients for the *n*-alkanes, 2-alkanones, and fatty acid methyl esters were fitted to eqn. 4

$$\log K_L = C + D(n) \tag{4}$$

where C and D are coefficients fitted by linear regression and n is the number of carbon

TABLE III

GAS–LIQUID PARTITION COEFFICIENTS FOR McREYNOLDS TEST SOLUTES ON TETRAALKYLAMMONIUM SULFONATE SALTS AT 121°C

Salt	*Test solute*						
	Benzene	*Butanol*	*2-Pentanone*	*Nitropropane*	*2-Methyl-2-pentanol*	*2-Octyne*	*Dioxane*
QBA MS	28.5(0.5)[a]	408.8(11.0)	42.0(0.2)	196.5(4.0)	226.1(5.6)	32.0(0.5)	48.7(0.9)
QBA FMS	25.4(1.9)	97.8(0.2)	48.3(0.4)	149.0(3.2)	70.4(0.8)	16.5(1.0)	50.0(0.2)
QBA BuS	24.2(1.4)	183.9(6.4)	31.5(2.0)	122.8(2.9)	183.9(6.4)	25.8(0.5)	33.4(1.0)
QBA FBuS	19.1(1.2)	82.7(5.5)	40.8(0.1)	128.8(5.0)	65.0(1.0)	25.9(0.6)	35.7(0.2)
QBA HS	29.2(1.3)	432.9(8.4)	44.6(0.9)	187.9(4.0)	259.8(5.4)	45.9(1.0)	47.0(0.9)
QBA OS	27.6(0.4)	408.0(20.0)	41.1(1.0)	155.4(1.1)	210.2(2.3)	46.9(1.0)	38.8(0.9)
QBA FOS	19.7(0.5)	67.3(2.1)	44.6(5.6)	106.0(1.5)	63.0(1.9)	32.3(0.9)	46.6(2.0)
QBA BS	23.9(1.1)	230.0(8.8)	37.1(1.0)	159.4(2.8)	126.3(4.5)	26.5(1.1)	44.8(1.7)
QBA FBS	23.1(0.7)	102.6(5.0)	40.8(0.8)	156.5(24.4)	65.8(2.0)	27.0(0.8)	41.2(2.1)
TBMA BuS	31.6(0.6)	687.4(20.0)	50.2(0.7)	211.2(2.2)	331.6(14.2)	38.3(0.8)	58.6(0.7)
TBMA FBuS	24.9(0.3)	96.4(1.2)	54.4(1.0)	160.8(10.8)	76.2(0.1)	28.3(0.7)	46.5(0.8)
TBMA HS	23.4(0.5)	333.6(8.3)	33.8(0.4)	132.3(2.8)	195.7(4.1)	34.5(0.5)	38.0(0.9)
TBMA OS	24.7(0.6)	383.1(9.2)	34.3(0.7)	138.5(5.0)	218.2(2.8)	38.7(1.3)	38.2(0.4)
TBMA FOS	16.8(1.2)	83.3(0.01)	47.5(0.6)	117.4(6.1)	71.9(0.5)	27.5(0.8)	39.8(3.3)
Squalane	32.1(0.9)	18.1(0.9)	26.4(0.3)	31.3(0.7)	39.1(0.6)	114.8(1.7)	31.4(0.4)

[a] Number in parentheses denotes the standard deviation.

atoms for the *n*-alkanes and the number of carbon atoms minus 2 for the 2-alkanones and fatty acid methyl esters. Coefficients obtained by linear regression are summarized in Table IV.

TABLE IV

COEFFICIENTS FOR EQN. 4

Salt	*Homologous series*	*Regression coefficients*			*Range (n)*
		C	*D*	r^2	
QBA MS	Alkanes	−0.6120	0.2094	1.00	13–16
	2-Alkanones	0.9881	0.2173	1.00	4–6
	FAME	1.0770	0..2124	1.00	5–8
QBA FMS	Alkanes	−0.6091	0.2040	1.00	13–15
	2-Alkanones	1.1192	0.2078	1.00	5–7
	FAME	1.0674	0.2113	1.00	4–7
QBA BuS	Alkanes	−0.4768	0.2114	1.00	13–16
	2-Alkanones	1.0339	0.2256	1.00	5–8
	FAME	1.1229	0.2211	1.00	5–7
QBA FBuS	Alkanes	−0.5548	0.2075	1.00	13–16
	2-Alkanones	0.9912	0.2180	1.00	4–7
	FAME	1.0863	0.2211	1.00	5–8
QBA HS	Alkanes	−0.4644	0.2257	1.00	13–17
	2-Alkanones	1.1005	0.2319	1.00	5–9
	FAME	0.9506	0.2416	1.00	4–7
QBA OS	Alkanes	−0.4987	0.2331	1.00	13–16
	2-Alkanones	0.8609	0.2491	1.00	5–7
	FAME	1.0017	0.2451	1.00	5–8
QBA FOS	Alkanes	−0.3860	0.2045	1.00	13–16
	2-Alkanones	1.0490	0.2144	1.00	5–7
	FAME	0.9242	0.2217	1.00	5–8
QBA BS	Alkanes	−0.6431	0.2031	1.00	13–16
	2-Alkanones	0.9347	0.2139	1.00	4–7
	FAME	0.7899	0.2097	1.00	5–8
QBA FBS	Alkanes	−0.6726	0.2170	1.00	13–16
	2-Alkanones	0.9469	0.2297	1.00	4–7
	FAME	0.8234	0.2241	1.00	5–8
TBMA BuS	Alkanes	−0.5238	0.2077	1.00	13–16
	2-Alkanones	1.0750	0.2132	1.00	4–7
	FAME	1.1740	0.2071	1.00	6–9
TBMA FBuS	Alkanes	−0.4372	0.1952	1.00	13–16
	2-Alkanones	1.1390	0.2042	1.00	5–7
	FAME	1.2070	0.1992	1.00	6–8
TBMA HS	Alkanes	−0.5735	0.2202	1.00	13–17
	2-Alkanones	0.8440	0.2340	1.00	4–7
	FAME	0.9945	0.2265	1.00	5–9
TBMA OS	Alkanes	−0.5886	0.2305	1.00	13–16
	2-Alkanones	0.8242	0.2449	1.00	4–6
	FAME	1.0156	0.2336	1.00	6–9
TBMA FOS	Alkanes	−0.4900	0.2079	1.00	13–16
	2-Alkanones	0.9188	0.2329	1.00	6–8
	FAME	1.1760	0.2040	1.00	6–9

The partial molar Gibbs free energy of solution for a methylene group was calculated according to eqn. 5

$$\Delta G_K^0(CH_2)^P = -2.3RT_cD_P \quad (5)$$

where $\Delta G_K^0(CH_2)^P$ is the partial molar Gibbs free energy of solution for a methylene group on phase P, R the universal gas constant (1.987 cal mol^{-1} K^{-1}), and D_P the regression coefficient for phase P defined in eqn. 4. In a similar way the partial molar Gibbs free energy of solution was calculated according to eqn. 6

$$(\Delta G_K^0X)^P = -2.3RT_c \log K_L^X \quad (6)$$

where $(\Delta G_K^0X)^P$ is the partial molar Gibbs free energy of solution for solute X on phase P with a gas–liquid partition coefficient K_L^X. The difference in free energies for solute X on two compared phases, one of which is the non-polar reference phase squalane, is given by eqn. 7

$$\delta(\Delta G_K^0X)_{SQ}^P = (\Delta G_K^0X)^P - (\Delta G_K^0X)^{SQ} \quad (7)$$

where $\delta(\Delta G_K^0X)_{SQ}^P$ is the difference in partial molar Gibbs free energy of solution for solute X on stationary phase P and squalane, SQ[23–26].

The activity coefficients at infinite dilution for the test solutes in the tetraalkylammonium sulfonate salts were calculated from eqn. 8

$$\gamma^0 = \frac{62\,370\rho T_c}{MP^0K_L} \quad (8)$$

where γ^0 is the approximate infinite dilution activity coefficient uncorrected for solute fugacity and gas phase non-ideality, ρ the solvent density at the column temperature T_c, M the molecular weight of the salt, and P^0 is the solute saturation vapor pressure at T_c obtained from tables in ref. 27.

RESULTS AND DISCUSSION

The tetraalkylammonium sulfonate salts are easy to prepare in high yield for the perfluoroalkanesulfonate salts (*ca.* 90%) and moderate yield for the alkanesulfonate salts (*ca.* 40%). Both series of salts are air stable compounds although the alkanesulfonates are hygroscopic, a property not shared by the perfluoroalkanesulfonate salts. The salts have favorable liquid ranges for GC, exceeding 100°C in all cases, with upper column temperature limits in the range 160–220°C (Table V). The greater density (Table II), higher melting points and greater thermal stability of perfluoroalkane compounds over their alkane analogues is a general characteristic feature of perfluorocarbon compounds[28]. All salts exhibited good column efficiencies with average values similar to those of conventional non-ionic phases prepared from the same batch of support. A typical test chromatogram for a mixture of 2-alkanones and acetophenone on QBA BuS and QBA FBuS is shown in Fig. 1.

TABLE V

USEFUL TEMPERATURE OPERATING RANGE FOR THE TETRAALKYLAMMONIUM SULFONATE SALTS

Salt	*Melting point (°C)*	*Column temperature limit (°C)*[a]	*Liquid range (°C)*[b]
QBA MS	78.5–79.5	180	101
QBA FMS	111.5–112.5	240	128
QBA BuS	34.5–35.5	160	130
QBA FBuS	44.5–45.5	220	175
QBA HS	Gel	170	(150)
QBA OS	Liquid	180	(160)
QBA FOS	Liquid	220	(200)
QBA BS	77.5–78.5	210	132
QBA FBS	51.5–52.5	210	158
TBMA BuS	44.5–45.5	160	115
TBMA FBuS	71.5–72.5	200	128
TBMA HS	49.5–50.5	170	120
TBMA OS	Gel	200	(180)
TBMA FOS	92.5–93.5	210	−117

[a] Defined as the highest temperature that the column could be maintained at for 24 h without change in retention or peak shape in a test chromatogram obtained at a lower temperature before and after the conditioning period.

[b] Values in parentheses assume a minimum operating temperature of room temperature (20°C) although these salts remain useable below room temperature.

The introduction of fluorine into the alkanesulfonate anion can influence retention in two ways. By the difference in interaction between solutes and the alkane and perfluoroalkane chains and by differences in the electron density on the sulfonate group resulting from the differences in inductive effects of the perfluoroalkane and alkane chains. These affects may not work in concert and should influence retention in a solute specific manner. For example, for alkanes that are retained largely by dispersive and inductive interactions there is a substantial reduction in retention on the QBA FOS salt compared to the QBA OS salt (Fig. 2). Assuming a partition model this must reflect a reduction in the dispersive interactions between *n*-alkanes and perfluoroalkanesulfonate salts compared to the analogous alkanesulfonate salts. Polar and hydrogen bond donor–acceptor solutes are influenced by changes in dispersive interactions and changes in specific solute–solvent interactions which should be more significantly influenced by the inductive effect of fluorine. For example, there are substantial changes in retention for a polar mixture on similar columns of QBA BuS and QBA FBuS (Fig. 3). An understanding of the above changes in terms of solute solvent interactions requires a more quantitative mechanistic treatment than is possible by comparing relative retention times of solutes on similar columns prepared with different salts.

The mechanism by which solutes are retained in gas–liquid chromatography can be either exclusively partitioning, exclusively adsorption or a mechanism in which both processes operate concurrently[22,25,26]. The gas–liquid partition coefficient, indepen-

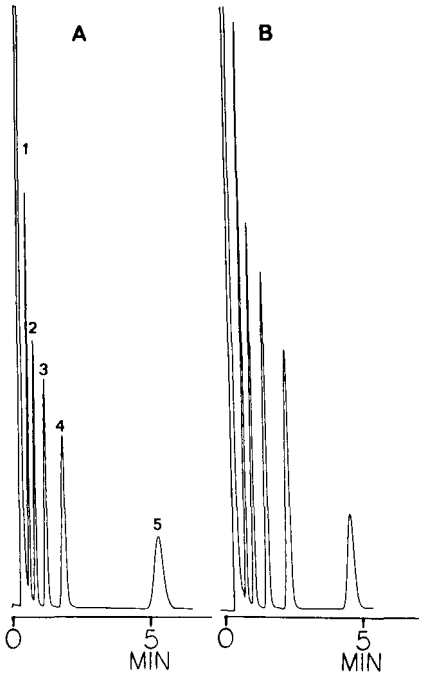

Fig. 1. Separation of a test mixture of ketones on 3.5 m × 2 mm I.D. columns of QBA BuS (A) and QBA FBuS (B) with 6% (w/w) loading of salt on Chromosorb W-AW (40–60 mesh) at 121°C with a nitrogen carrier gas flow-rate of 11.0 ml/min. Peak identification: 1 = hexan-2-one; 2 = heptan-2-one; 3 = octan-2-one; 4 = nonan-2-one; 5 = acetophenone.

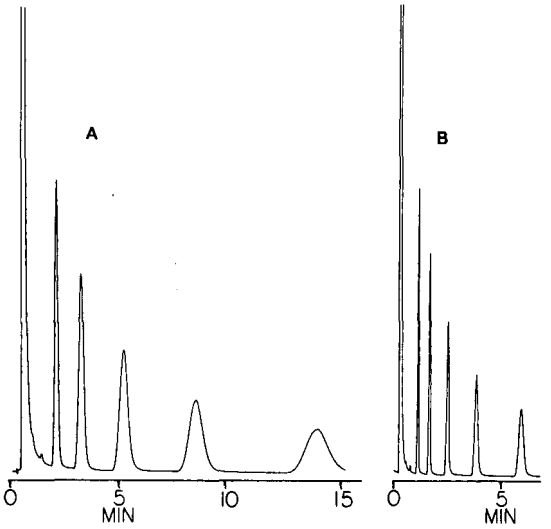

Fig. 2. Separation of a test mixture of *n*-alkanes (C_{13}–C_{17}) on matched columns of (A) QBA OS and (B) QBA FOS. Column 3.5 m × 2 mm I.D. with 8.0% (w/w) salt on Chromosorb W-AW (40–60 mesh) at 121°C with a nitrogen carrier gas flow-rate of 17.0 ml/min.

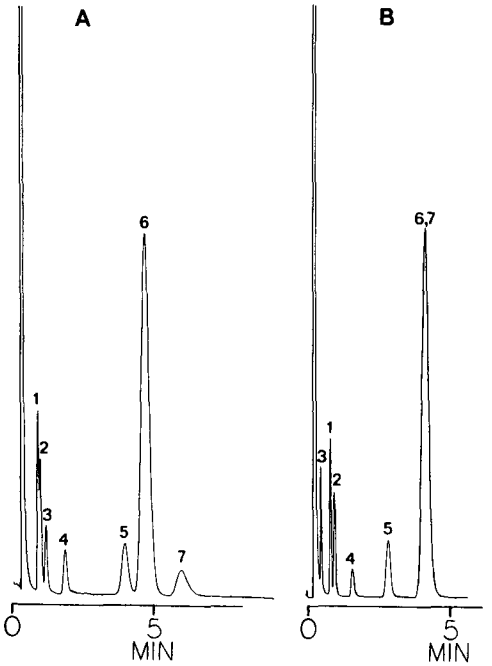

Fig. 3. Separation of a polar test mixture on matched columns of (A) QBA BuS and (B) QBA FBuS. Column 3.5 m × 2 mm I.D. with 6.0% (w/w) salt on Chromosorb W-AW (40–60 mesh) at 131°C with a nitrogen carrier gas flow-rate of 11.0 ml/min. Peak identification: 1 = *n*-butylbenzene; 2 = dihexyl ether; 3 = 2-methyl-2-pentanol; 4 = 1-nitropentane; 5 = benzonitrile; 6 = naphthalene; 7 = benzodioxane.

dent of contributions to retention arising from interfacial adsorption, can be obtained from plots of V_N^*/V_L *vs.* $1/V_L$ according to eqn. 3 and a qualitative estimate of the importance of interfacial adsorption obtained from the slopes. Of the alkanesulfonate and perfluoroalkanesulfonate salts selected for study only QBA MS, QBA FMS, QBA HS, TBMA BuS, and TBMA FBuS retained a wide range of different solutes exclusively by gas–liquid partitioning. In all other cases retention occurs by a combination of partitioning and interfacial adsorption. The relative importance of interfacial adsorption as a retention mechanism depends on properties of both the solute and the salt and is not related in any manner to the incorporation of fluorine into the alkanesulfonate group. Interfacial adsorption seems to be more important for the arylsulfonate salts (Fig. 4) than for the alkanesulfonate salts for which the data for QBA BuS and QBA FBuS (Fig. 5) are fairly representative. Since experimental data for the interfacial areas required to evaluate the adsorption coefficients in eqn. 3 are unavailable and just about impossible to measure reliably[14,22,23], a quantitative indication of the importance of interfacial adsorption as a retention mechanism can be obtained by comparing the experimental specific retention volume with the specific retention volume calculated from the gas–liquid partition coefficient. The difference between these two values is the contribution made to retention attributable to interfacial adsorption. These data are collected in Table VI at two phase loadings corresponding to $1/V_L = 3$ [*ca.* 5% (w/w) salt] and $1/V_L = 8$ [*ca.* 15% (w/w)]. For an

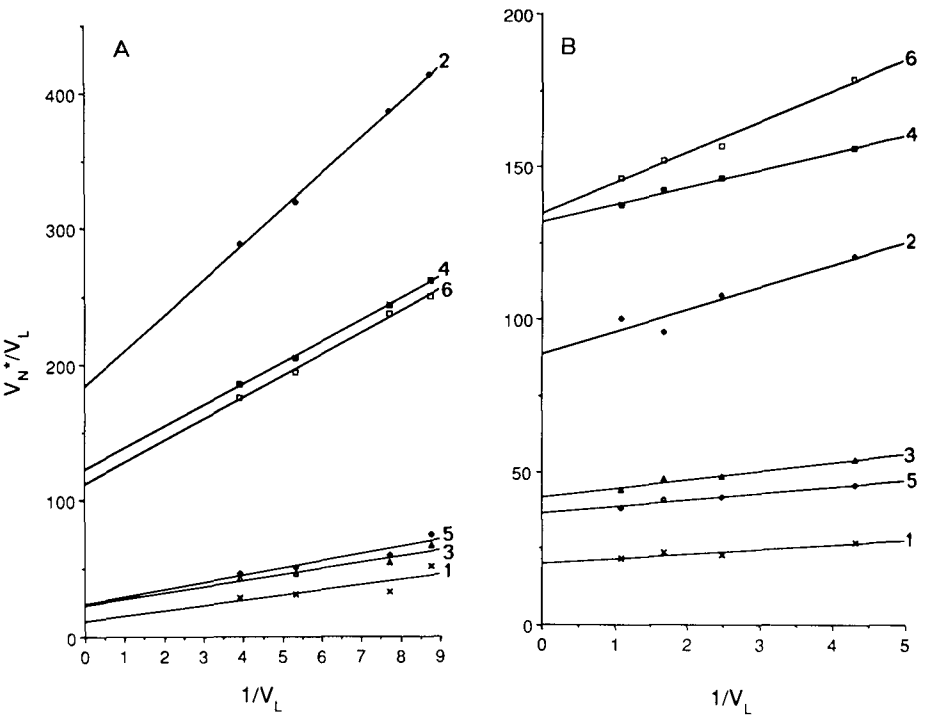

Fig. 4. Plot of V_N^*/V_L *vs.* $1/V_L$ for a series of test solutes on QBA BS (A) and QBA FBS (B). Solute identification: 1 = benzene; 2 = butanol; 3 = 2-pentanone; 4 = 1-nitropropane; 5 = dioxane; 6 = tridecane.

exclusively gas–liquid partitioning mechanism the specific retention volume is independent of the volume of liquid phase but this is not the case when interfacial adsorption also contributes to retention. Interfacial adsorption should be less important at high phase loadings when retention occurs by a mixed mechanism since the ratio of the phase volume to surface area is higher and the liquid surface area, which does not change linearly with V_L, declines due to preferential filling of the micropores of the support ahead of the macropores[23]. This behavior is clearly seen in the data in Table VI. The contribution made by interfacial adsorption is very variable but in several cases is clearly significant, accounting for the greater part of the observed experimental specific retention volume. There is no general trend, however, attributable to the incorporation of fluorine into the alkanesulfonate anion as far as the relative importance of partitioning and interfacial adsorption are concerned. In most cases though the observed retention [V_g(Ex)] and that portion of the observed experimental retention that is attributable to gas–liquid partitioning [$V_g(K_L)$] is smaller for the perfluoroalkanesulfonate anions than their alkanesulfonate analogues.

The influence of anion chain length on the partitioning component of the retention volume can be discerned from plots of K_L as a function of the carbon number of the anion. When the test solutes are *n*-alkanes (Fig. 6) there is a linear relationship between K_L and the number of carbon atoms substituted with hydrogen or fluorine in the anion. Both homologous series of anions show a positive slope with the slope for

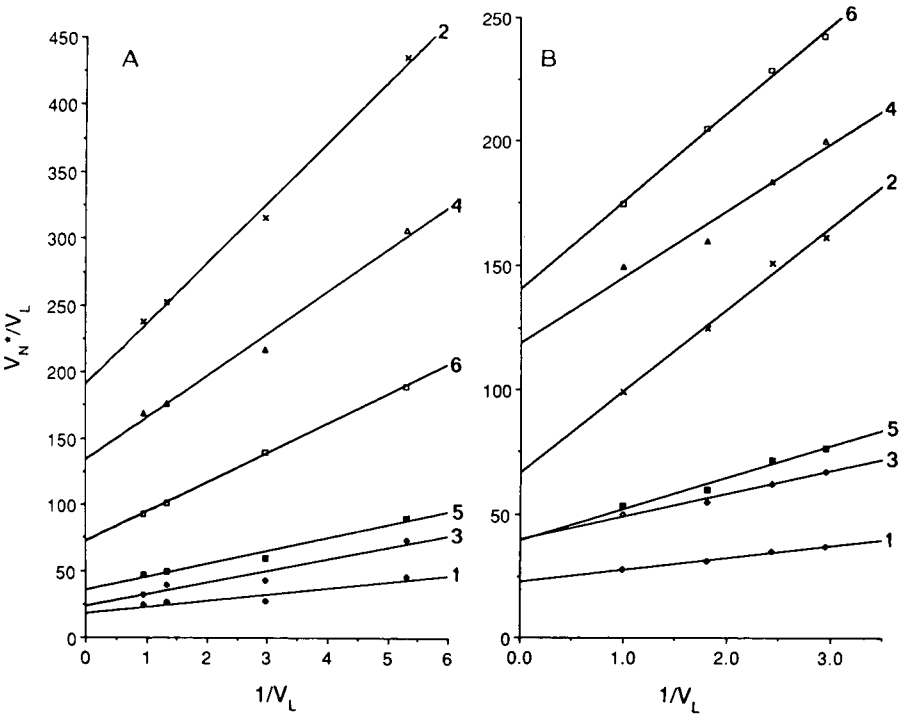

Fig. 5. Plot of V_N^*/V_L *vs.* $1/V_L$ for a series of test solutes on QBA BuS (A) and QBA FBuS (B). Solutes are identified in Fig. 4.

the perfluoroalkanesulfonates being significantly smaller than that of the alkanesulfonates. Retention increases with increasing chain length and the lowest retention of the *n*-alkanes is observed for the methanesulfonate and trifluoromethanesulfonate anions. The difference in the partial molar Gibbs free energy of solution per methylene group, Table VII, for transfer between a tetrabutylammonium alkanesulfonate and perfluoroalkanesulfonate corresponds to 12, 20, and 59 cal/mol for the C_1, C_4, and C_8 anion sidechains, respectively. This is in good agreement with the values for the tributylmethylammonium salts of 18 and 59 cal/mol for a C_4 and C_8 sidechain, respectively.

For polar test solutes the change in K_L as a function of anion chain length is less well defined. The alkanesulfonates show either little change or a great deal of scatter around what seems to be a mean value. The perfluoroalkanesulfonate salts show similar behavior or a decrease in the value of K_L with increasing chain length. This difference in behavior of the *n*-alkanes and polar test solutes is most likely due to differences in the importance of selective solute–solvent interactions which are influenced by the inductive effect of fluorine as well as by its influence on non-specific solute–solvent interactions. The behavior of the *n*-alkane standards can not be used as a reliable measure of the behavior of polar test solutes or to predict the general trends concerning the importance of chain length on retention.

Differences in selectivity accompanying the replacement of hydrogen by fluorine in the alkanesulfonate anions are discernible from differences in the values for

TABLE VI

CONTRIBUTION OF INTERFACIAL ADSORPTION TO RETENTION

$V_g(K_L)$ = Specific retention volume calculated from the gas–liquid partition coefficient. $V_g(Ex)$ = Experimentally determined value of the specific retention volume at a specific value of V_L. $\Delta\% = \{[V_g(Ex) - V_g(K_L)]/V_g(K_L)\} \cdot 100\%$.

Salt	$1/V_L$	*Test solute*								
		Tridecane			*Ethylbenzene*			*2-Methyl-2-pentanol*		
		$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$	$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$	$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$
QBA BuS	3	151.7	151.7	0	41.6	51.2	23		194.4	42
	8					67.1	61	136.6	291.4	113
QBA FBuS	3	86.7	100.6	16	32.0	39.7	24	40.6	48.5	19
	8		124.0	43		51.0	59		61.7	52
QBA OS	3	247.5	247.5	0	59.5	59.5	0	153.2	189.7	24
	8								252.9	65
QBA FOS	3	98.8	98.8	0	29.1	29.7	2	33.2	33.2	0
	8					33.0	13		41.4	25
QBA BS	3	67.8	67.8	0		43.7	9	86.4	99.9	16
	8				40.2	48.0	19		125.8	46
QBA FBS	3	86.1	151.3	76	37.9	60.9	61	40.4	76.0	88
	8		259.9	202		99.3	162		135.5	235
TBMA OS	3	191.0	226.7	19	53.1	58.9	11	162.8	177.9	9
	8		281.6	47		68.9	30		202.3	24
TBMA FOS	3	84.8	87.3	3	30.5	30.6	0	37.3	37.7	1
	8		91.7	8		30.8	0		38.6	3
		Octanone			*Nitropropane*			*Dioxane*		
		$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$	$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$	$V_g(K_L)$	$V_g(Ex)$	$\Delta\%$
QBA BuS	3			0	91.2	126.1	38	24.8	30.5	23
	8	181.4	181.4			184.7	103		49.5	100
QBA FBuS	3	125.2	145.1	16	80.0	87.0	8	22.3	26.8	20
	8		176.6	41		100.2	24		34.0	52
QBA OS	3	166.2	187.6	13	113.3	126.0	11	28.3	35.2	24
	8		218.5	31		147.9	31		46.8	65
QBA FOS	3	114.4	114.4	0	55.9	55.9	0	24.6	24.6	0
	8									
QBA BS	3	113.3	132.7	17	109.0	132.1	21	30.7	36.1	18
	8		169.3	49		172.8	59		46.6	52
QBA FBS	3	131.0	211.9	62	96.1	96.1	0	25.3	47.2	87
	8		346.6	165					83.8	231
TBMA OS	3	146.8	164.3	12	103.4	103.4	0	28.5	33.2	16
	8		193.3	32					41.3	45
TBMA FOS	3	107.2	110.5	3	60.9	60.9	0	20.6	20.6	0
	8		116.2	8						

$\delta(\Delta G^0_K X)^P_{SQ}$ where X represents different test solutes selected to express specific molecular interactions (the McReynolds test solutes)[22–26,29]. The values of $\delta(\Delta G^0_K X)^P_{SQ}$ for benzene and 2-octyne are all positive indicating greater solubility in the non-selective reference phase, squalane, than in any of the salts. In agreement with the previous discussion these solutes show stronger interactions with the alkanesulfonate

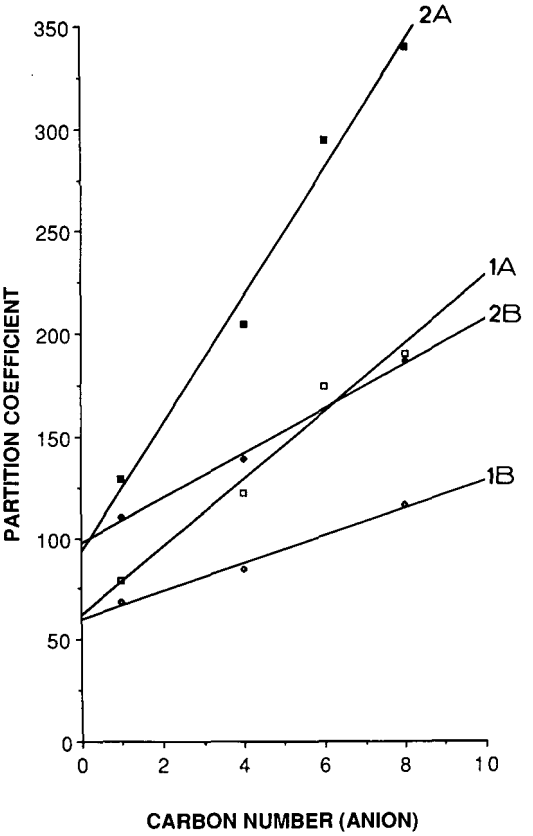

Fig. 6. Plots of the gas–liquid partition coefficient as a function of the number of carbon atoms in the anion chain for dodecane (1) and tridecane (2) on the tetrabutylammonium alkanesulfonates (A) and perfluoroalkanesulfonates (B) at 121.2°C.

salts than with the perfluoroalkanesulfonate analogues. The proton donor solutes butanol and 2-methyl-2-pentanol are strongly retained by all salts; more strongly by the alkanesulfonates than the perfluoroalkanesulfonate analogues. The differences in the $\delta(\Delta G_K^0 X)_{SQ}^P$ values in this case are quite large, generally greater than 1 kcal/mol, and it seems reasonable to speculate that this must result in large part from a reduction in the electron density on the sulfonate group caused by the inductive effect of the perfluoroalkane group. Substantial differences in the retention of alcohols on alkanesulfonates and perfluoroalkanesulfonates will occur, therefore, on matched columns with the alcohols showing diminished retention on the perfluoroalkanesulfonate salts. In the case of the electron donor solutes, 2-pentanone and dioxane, an increase in retention on the perfluoroalkanesulfonates compared to their alkanesulfonate analogues was observed. These differences are generally not very great but must occur in opposition to any reduction in the magnitude of non-specific interactions. Since the salts contain no proton donor groups it can be assumed that 2-pentanone and dioxane are selectively retained on the perfluoroalkanesulfonates by electron donation to the electron deficient group or carbon backbone of the

TABLE VII

CHANGES IN SOLVENT STRENGTH AND SELECTIVITY ACCOMPANYING THE INTRODUCTION OF PERFLUOROCARBON GROUPS INTO TETRAALKYLAMMONIUM SULFONATE SALTS

Salt	$\Delta G_K^0(CH_2)$	$\delta(\Delta G_K^0 X)_{SQ}^P$						
		Benzene	*Butanol*	*2-Pentanone*	*1-Nitropropane*	*2-Methyl-2-pentanol*	*2-Octyne*	*Dioxane*
QBA MS	−486	0.092	−2.443	−0.363	−1.440	−1.376	1.000	−0.344
QBA FMS	−474	0.182	−1.322	−0.473	−1.223	−0.461	1.150	−0.365
QBA BuS	−505	0.219	−1.817	−0.138	−1.071	−1.214	1.170	−0.048
QBA FBuS	−485	0.405	−1.191	−0.340	−1.109	−0.399	1.167	−0.101
QBA HS	−412	0.073	−2.488	−0.410	−1.405	−1.263	0.942	−0.316
QBA OS	−541	0.120	−2.442	−0.346	−1.256	−1.319	0.701	−0.166
QBA FOS	−482	0.383	−1.030	−0.410	−0.956	−0.374	0.994	−0.309
QBA BS	−467	0.229	−1.993	−0.265	−1.276	−0.919	1.150	−0.279
QBA FBS	−499	0.257	−1.360	−0.341	−1.262	−0.408	1.135	−0.212
TBMA BuS	−469	0.012	−2.850	−0.502	−1.497	−1.676	0.861	−0.489
TBMA FBuS	−451	0.198	−1.310	−0.565	−1.283	−0.523	1.097	−0.308
TBMA OS	−525	0.204	−2.392	−0.205	−1.166	−1.348	0.852	−0.155
TBMA FOS	−466	0.509	−1.196	−0.469	−1.036	−0.478	1.119	−0.185
TBMA HS	−402	0.073	−2.284	−0.192	−1.130	−1.485	0.942	−0.150

perfluoroalkanesulfonate anions resulting from the inductive effect of fluorine. For the dipole probe 1-nitropropane the $\delta(\Delta G_K^0X)_{SQ}^P$ values are generally more favorable for the alkanesulfonates but differences between the alkanesulfonates and perfluoroalkanesulfonates are generally quite small. The inductive effect of fluorine does not have a large influence on orientation interactions which are, presumably, mainly dominated by the Coulombic fields between ions.

Activity coefficients for the test solutes in the alkanesulfonate and perfluoroalkanesulfonate salts are summarized in Table VIII. These are uncorrected for solute fugacity and non-ideality of the gas phase and should only be considered as approximate. However, they are sufficiently accurate to illustrate some interesting differences in the solution characteristics of the different anion types. Dodecane shows a large deviation from regular solution behavior but this is due mainly to its low vapor pressure at the measurement temperature. In general, the solution of non-polar solutes in the perfluoroalkanesulfonate salts shows a greater deviation from ideality than the dissolution of the same solute in the alkanesulfonate salt. This is in general agreement with the fact that *n*-alkane and perfluoroalkane solvents are only partially miscible and although not differing greatly in polarity they do not form ideal solutions[28]. When the solute becomes polarizable, in the case of benzene, solution in the perfluoroalkanesulfonates is more ideal than in the case of the alkanesulfonates. Butanol shows similar behavior with the differences in activity coefficients between the alkanesulfonate and perfluoroalkanesulfonates being much larger. The opposite general behavior is seen for the electron donor solutes confirming that deviations from ideal solution behavior result from specific solute–solvent interactions, to a large extent, and these are influenced primarily by the inductive effect of fluorine.

In conclusion, the tetraalkylammonium sulfonate salts are useful stationary phases possessing wide liquid ranges and good thermal stability. The introduction of fluorine into the alkanesulfonate anion has a significant influence on the selectivity of

TABLE VIII

APPROXIMATE ACTIVITY COEFFICIENTS AT 121.2°C FOR DIFFERENT TEST SOLUTES IN THE ALKANESULFONATE AND PERFLUOROALKANESULFONATE SALTS

Salt	*Test solute*					
	Dodecane	*Benzene*	*Butanol*	*2-Pentanone*	*1-Nitropropane*	*Dioxane*
QBA MS	19.88	0.86	0.20	1.26	0.63	1.07
QBA FMS	21.45	0.90	0.77	1.03	0.78	0.97
QBA BuS	11.20	0.88	0.38	1.46	0.88	1.48
QBA FBuS	13.48	0.91	0.70	0.94	0.70	1.05
QBA OS	6.38	0.69	0.15	1.00	0.62	1.03
QBA FOS	8.45	0.78	0.75	0.75	0.74	0.70
QBA BS	23.00	0.92	0.31	1.29	0.70	1.04
QBA FBS	15.11	0.86	0.64	1.06	0.65	1.03
TBMA BuS	18.05	0.69	0.12	1.05	0.58	0.88
TBMA FBuS	17.12	0.84	0.71	0.83	0.66	0.95
TBMA OS	8.82	0.83	0.18	1.29	0.75	1.14
TBMA FOS	10.40	0.99	0.59	0.76	0.72	0.88

the salts as well as acting to reduce the importance of non-specific interactions. The inductive effect of fluorine acts to reduce the retention of proton donor solutes such as alcohols and to increase the retention of electron donor solutes such as ketones and ethers. These competing factors must be considered when attempting to explain the observed retention of solutes on alkane- and perfluoroalkanesulfonate salts. The contribution to retention from interfacial adsorption cannot be ignored as a retention mechanism for some salts. Interfacial adsorption is not a characteristic property of a particular anion type, alkane- or perfluoroalkanesulfonate, and its relative importance cannot simply be estimated from a knowledge of anion structure.

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CHROMSYMP. 1555

HIGHLY SENSITIVE ON-LINE RADIOASSAY OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC EFFLUENTS

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SUMMARY

We previously described a procedure for highly sensitive radiochromatography that is based on depositing fractions of the high-performance liquid chromatographic effluent on non-wetting film, concentrating them, transferring them to filter paper, generating autoradiographs of the paper on photographic film, and quantifying by densitometry. The work reported here included design of: a modified procedure for assaying non-polar compounds in which the transfer solvent was changed and silica gel thin-layer chromatography plates were substituted for the filter paper; a method for detecting tritium by adding scintillator to the filter paper before autoradiography and for distinguishing ^{14}C and ^{3}H by comparing autoradiographs with and without added scintillator. Then, to decrease the time for assaying the fractions, we tested two different kinds of gas radiation detectors, using the equivalent of an array of detectors working simultaneously. Since these detectors permitted a number of fractions to be assayed at the same time, the time each was assayed could be extended and/or the same number of fractions could be assayed more rapidly.

Following the successful application of these detector arrays, we designed a system concept for using an array of detectors for increasing the sensitivity of on-line, flow-through detection. Fractions of the effluent are passed through a series of detectors sequentially. The counting rates of each counter for each time interval are recorded. The results are summed electronically to reflect the position of each sample in the array. The same resolution is achieved as in flow-through detection, with comparable convenience but much enhanced sensitivity, since each fraction is assayed for a much longer time.

INTRODUCTION

Because of the random nature of radioactive decay, the precision of any radioassay is a function of the number of radioactive events that contribute to the measurement. For any prescribed precision, the sample must therefore remain in the active volume of the detection system for the time needed for the required number of disintegrations to be detected. In radiochromatography the precision is related to the time each portion of the chromatogram or chromatographic effluent remains in the active volume of the detector. With a flow-through detector, time in the detector must

0021-9673/89/$03.50

be limited if the resolution of the chromatography is to be preserved. This limits the sensitivity of radiochromatography in on-line high-performance liquid chromatography (HPLC). If less radioactivity is present than can be measured with acceptable precision in up to *ca.* 20 s, flow-through detection is generally not useful. The usual alternative is to fractionate the effluent for subsequent radioassay. Here again, preserving the resolution of the chromatography requires that separate fractions be collected at least every 20 s. Many fractions must then be assayed, and this can occupy a counting device for many hours. For this reason, most workers find flow-through detectors more convenient to use and reserve fraction-collecting for those assays involving very low levels of radioactivity. Even then, most people tend either to limit the time each fraction is counted or to sacrifice resolution for sensitivity by collecting fewer fractions.

In work described previously, we attempted to address this problem by devising methods for making fraction collection more convenient and assaying their radioactivity more sensitive[1]. We found that we could deposit equal, small portions of HPLC effluents on non-wetting fluorocarbon film, concentrate each fraction to near dryness, transfer the fractions to filter paper where they form uniform 2-mm-diameter circular spots, and then assay all the spots for radioactivity simultaneously by autoradiography on photographic film followed by densitometry. ^{14}C at levels of > 50 dpm per fraction was measurable by an overnight exposure. We adapted an automatic fraction collector for this technology and could collect 80 equal fractions, reproducing in the record of the radioactive analysis the UV absorbance or fluorescence elution patterns[2].

This procedure proved less suitable for assay of water-insoluble compounds because they did not procedure spots on filter paper with uniform distribution of radioactivity, and therefore densitometry of the radioautograph was less quantitative.

In the first part of the work described here, we modified the procedure to make it more suitable for non-polar compounds by changing the transfer solvent and substituting silica gel thin-layer chromatography (TLC) plates for the filter paper. We detected ^{3}H by adding scintillator to the filter paper before the autoradiography and could distinguish ^{14}C and ^{3}H by comparing autoradiograms with and without added scintillator. Then, to decrease the time for assaying the fractions, we tested two diffeent kinds of gas radiation detectors, using the equivalent of an array of detectors working simultaneously. We considered the photographic film used in autoradiography to be similar in essence to a large number of radiation detectors operating simultaneously. Since the array of detectors permitted a number of fractions to be assayed at the same time, the time each was assayed could be extended and/or the same number of fractions could be assayed more rapidly.

Finally, we designed a system concept for using an array of detectors for increasing the sensitivity of on-line, flow-through detection.

EXPERIMENTAL

Material

Prostaglandin standards were purchased from Biomol (Plymouth Meeting, PA, U.S.A.), arachidonic acid from Sigma (St. Louis, MO, U.S.A.), [^{3}H]prostaglandins from Amersham International (Arlington Heights, IL, U.S.A.) and [^{14}C]arachidonic

acid and En^{3}hance Spray from New England Nuclear (Boston, MA, U.S.A.). Plastic-backed silica gel 60 TLC sheets (20 × 20 cm, 0.2 mm layer thickness) were bought from E. Merck (Darmstadt, F.R.G.).

Equipment

HPLC of prostaglandin precursors and metabolites was performed with a Constametric III pump (LDC, Riviera Beach, FL, U.S.A.), a Model 85 LC detector (Perkin-Elmer, Norwalk, CT, U.S.A.) with a 2-μl flow cell and a 7120 injector valve (Rheodyne, Cotati, CA, U.S.A.) with a solvent by-pass loop. The column, 30 × 4.6 mm I.D., packed with 3-μm C_{18}-coated silica was bought from Perkin-Elmer. Control of the step gradient of the mobile phase from 32 to 75% acetonitrile in 0.002 *M* hydrochloric acid was achieved by the Model 420 gradient microprocessor (Altex, Berkeley, CA, U.S.A.). The gradient was held at 32% for the first 6 min and at 75% for the next 12 min. The flow-rate was at 0.5 to 1 ml/min. The absorbance of the column effluent was monitored at 210 nm; the amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.).

Fraction collection

The fraction collector is based on the fluorocarbon film technology used to concentrate samples and to apply them to TLC plates by Fenimore and Davis[3]. For these experiments the fractionator was adapted for use with a Gilson fraction collector. The tubes were replaced by a hollow rectangular aluminum plate, 20.8 × 6.4 cm, with 80 wells (16 columns and 5 rows), 0.9 mm deep, milled on the upper surface, each supplied with a 1-mm hole at the bottom. The top surface of the plate was polished to mirror finish. The plate was constructed by cementing the 3-mm plate, machined as described, to a 1-cm-wide silicone rubber gasket, 1 mm thick, around its perimeter, which in turn was cemented to a second aluminum plate, 1.5 mm thick, provided with fittings for applying vacuum or pressure to the space between the plates formed by the gasket. A 20 × 3.75 cm, 100-W strip heater (Rama Industrial Heater, San Jacinto, CA, U.S.A.) was fixed to the bottom plate. Fluorocarbon-coated sample wells were formed by placing a sheet of 0.025-mm-thick fluorocarbon film over the plate and applying vacuum to the cavity. The column effluent was delivered by the fraction collector arm to each well through 15 cm × 0.002 in. I.D. stainless-steel capillary tube, fitted with a 2-mm long 1 PTFE sleeve at the end. From 20 to 80 μl of HPLC effluent were deposited in each well. In assays of amino acids, the final volume of each fraction after evaporation was limited by including 1% ethylene glycol in the mobile phase. For assays of lipids, 25 μl of 0.6% oleic acid in ethanol was added to each well. After evaporation, the residues in the oleic acid carrier were transferred to a plastic-backed silica gel sheet, 200 × 70 × 0.2 mm, by replacing the vacuum with 10 p.s.i. nitrogen pressure.

An autoradiogram was prepared by clamping the silica gel sheet against X-ray film in a Wolf X-ray cassette, 20 × 30 cm, and placing it in a freezer at −75°C. For assaying tritiated compounds, the TLC sheet was sprayed with En^{3}hance Spray scintillator solution before exposing it to the film. The film was then developed, and densitometry was performed with a Model CS-910 TLC (Shimadzu, Columbia, MD, U.S.A.). The scanner record was processed on an HP-3390 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Position-sensitive proportional counter

The efficacy of scanning the collected fractions with a position-sensitive proportional counter was assessed by scanning the radiochromatogram of amino acids that had previously been autoradiographed. The scanner was a Model RS Radio TLC scanner (Radiomatic Instruments and Chemical Co., Tampa, FL, U.S.A.).

The counter itself is a 20-cm-long, windowless proportional counter, used with "P-10" gas, and connected to a 1024-channel multichannel analyzer. With its associated aperture, it offers 1- to 2-mm resolution of ^{14}C. For assaying TLC plates, the counter moves over the plate stepwise in small increments at predetermined counting intervals. When the β-particle interacts with the gas in the counter, an electric discharge occurs in the volume where the interaction occurs. This induces a charge in an inductive delay line close to the wire. The charge travels as a pulse to the ends of this delay line at measurable speed. The position where the event occurs is sensed electronically by the time of arrival of the pulse at each end of the delay line. For the scan of the filter paper described here, the wire was set to count for 5 min at each of the 16 rows of spots on the filter paper, advancing 12 mm to the next row of spots automatically at the end of that time. The output was recorded in several ways, including the graphic representation of the counting rate across the row, as well as the total counts recorded at each location.

A second, similar experiment was performed on the same radiochromatogram of amino acids with the Vanguard Model 2001 1-D/2-D TLC plate scanner (Digital Diagnostic, Hamden, CT, U.S.A.). This scanner uses an array of ten 1 × 2.5 cm windowless flow-counters, operated in the Geiger mode with "Q-gas". The counters are fitted with metal collimators to achieve the required resolution in TLC scanning. To scan the filter paper with spots 0.5 in. apart, a collimator offering 0.5-cm diameter apertures was used. The counts along 10 columns of 5 spots were recorded for 10 min each.

Procedure

Metabolism of arachidonic acid by human platelets. A platelet concentrate (*ca.* 10 ×) was centrifuged at 250 *g* for 15 min to remove residual red cells. The supernatant was recentrifuged at 1800 *g* for 15 min. The resulting platelet pellet was washed with 10 ml of Tris buffer (pH 7.0–7.2), containing 1.5 m*M* EDTA. The washed platelets were then resuspended in 2 ml of Tris buffer without EDTA. This platelet preparation was incubated with $9 \cdot 10^5$ dpm of [^{14}C]arachidonic acid for 20 min at 37°C. The incubation was stopped by the addition of 0.5 ml glacial acetic acid, sufficient to bring the pH to 3 and the incubate was extracted twice with 3-ml aliquots of ethyl acetate.

The extract was evaporated to dryness on the contact spotter, and the residue was redissolved in 200 μl of ethanol. A 6-μl volume of the extract, containing approximately 24 000 dpm was injected into the HPLC column.

RESULTS

On a column packed with 3-μm particles, the prostaglandin metabolites could be separated in 15 min with a resolution comparable to that achieved much more slowly on columns containing larger particles. The effluent could be delivered to the fraction collector in the short length of microbore tubing described with minimal loss of

resolution, as judged by the peak widths on the record of the radioassay and that of the UV absorbance detector. In the analysis of amino acids, glycerol was added to the mobile phase to limit the volume of fractions after evaporation. Glycerol kept both the amino acids and the residual buffer salts in solution and facilitated delivery of the condensed fractions to the filter paper matrix. The autoradiograms were circular spots of apparently uniform density. When the same procedure was tried for transferring the residues from the prostaglandin analysis, the autoradiographic images were not uniform in density: they contained what appeared to correspond to particles of precipitate on the filter paper. When oleic acid was substituted for the glycerol, on the basis that it was liquid, had limited volatility, and should dissolve prostaglandins, the spots were uniform in density but more diffused than with the glycerol.

Substitution of the plastic-backed silica TLC sheet for the paper in radioassay of [^{14}C]arachidonic acid with oleic acid on the TLC sheet, yielded uniformly dense spots on the X-ray film that reproduced the elution pattern obtained with the UV detector (Fig. 1).

With the new spotting technique, quantitative radioassays of [^{3}H]thromboxane B_2 and [^{14}C]arachidonic acid were obtained. The radioactivity was proportional to the density of the spot on the exposed film at both high and low activity. The exposure was 7 h for 2000–8000, and 184 and 92 h for 20–120 dpm [^{3}H]thromboxane and [^{14}C]arachidonic acid, respectively.

Autoradiograms of [^{3}H]prostaglandins and [^{14}C]eicosanoids showed that ^{3}H is virtually undetected unless the paper or TLC plate was sprayed with scintillator before exposing it to the film. Chromatograms of standards yielded good separations (Figs. 2 and 3). In analyses of the products of arachidonic acid metabolism there was some loss of resolution, but this was difficult to quantify because of the relatively poor resolution of the many apparent metabolites present (Figs. 4 and 5). [^{14}C]Arachidonic acid was metabolized to several radioactive products, including thromboxane B_2, prostaglandin $F_{2\alpha}$ and hydroxyeicosatetraenoic acids.

Scanning with the two kinds of gas detectors gave similar results. The scan of fractions from an analysis of three amino acids, containing 39 000 dpm, approximately

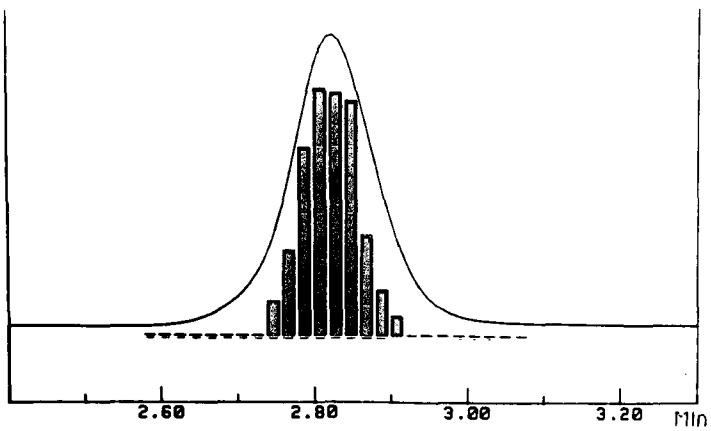

Fig. 1. Scans of the radioautographs (black bars) superimposed on the UV chromatogram of arachidonic acid.

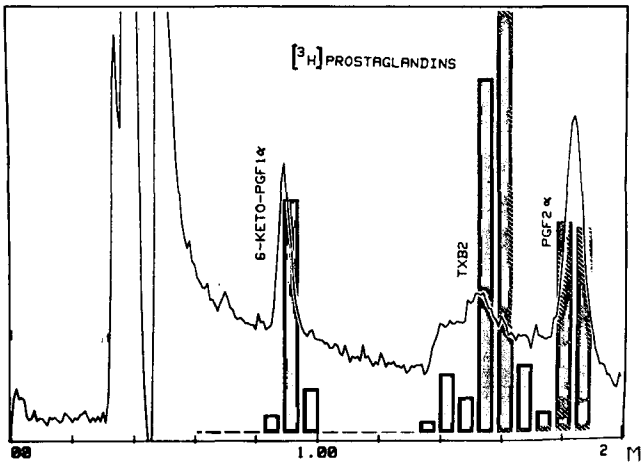

Fig. 2. Scans of the radioautographs superimposed on the UV chromatogram of the prostaglandins standards. Conditions: Perkin-Elmer reversed-phase C_{18} column 3 μm, 3 cm × 4.6 mm I.D. Mobile phase gradient from 32 to 75% acetonitrile in 0.002 *M* hydrochloric acid was controlled by the Altex Model 420 gradient microprocessor. The gradient was held at 32% for the first 6 min, and at 75% for the next 12 min. Flow-rate of the effluent was at 0.5 to 1 ml/min. The UV absorbance of the column effluent was monitored at 210 nm. 6-KETO-PGF 1α = 6-ketoprostaglandin $F_{1\alpha}$; PGF 2α = prostaglandin $F_{2\alpha}$; TXB2 = thromboxane B_2.

equally distributed among the three, gave a usable autoradiogram after 6 h of exposure to the film (Fig. 6). The maximum counting rate of an individual fraction was 1500 dpm. Counting the same fractions with the two gas counting systems gave gave usable results in less than 1 h of counting, even though the counting routines were not optimized.

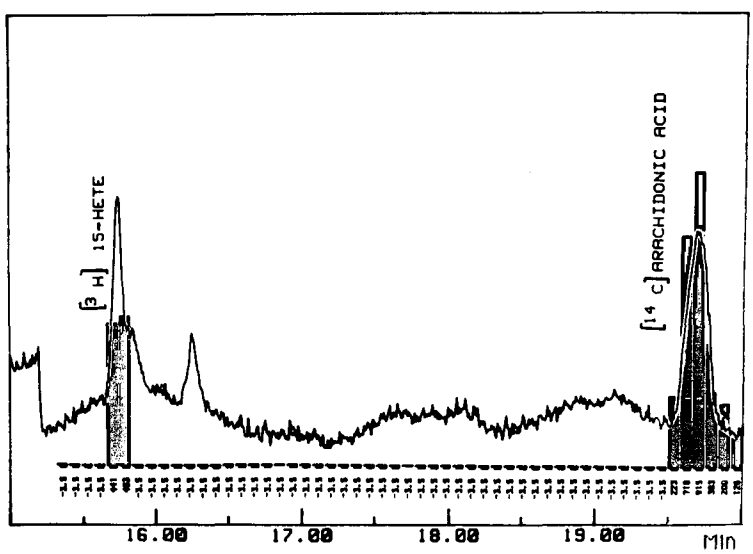

Fig. 3. Scans of the radioautographs superimposed on the UV chromatogram of the hydroxyeicosatetraenoic acid (15-HETE) and arachidonic acid. Conditions as in Fig. 2.

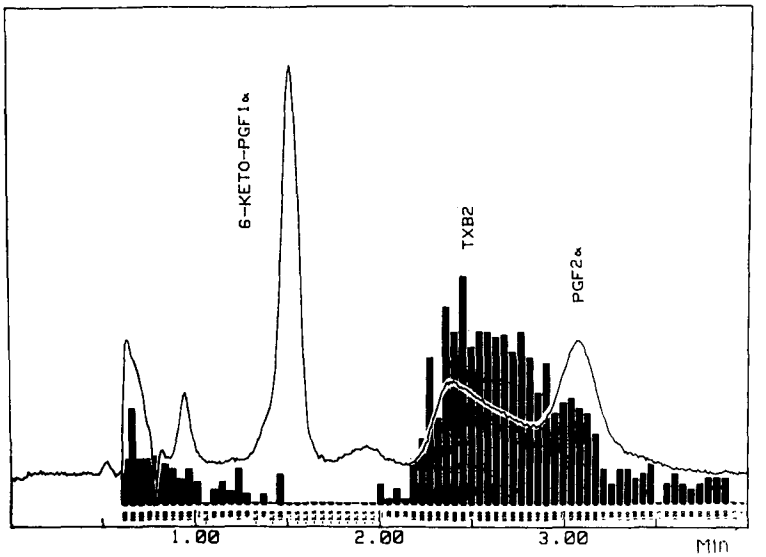

Fig. 4. Scans of radioautographs superimposed on the UV chromatogram of prostaglandin metabolites of platelets of arachidonic acid. Conditions as in Fig. 2.

The Geiger counters were 20 mm apart, and the fractions 12.7 mm (0.5 in.) apart, requiring that the counters scan the paper in order to cover the radioactive samples, thus spending at least part of the time away from the radioactivity. Allowing the counters to count for 10 min over each column of spots, with scanning, gave results that approximated those of the autoradiogram. The efficiency of the position-sensitive

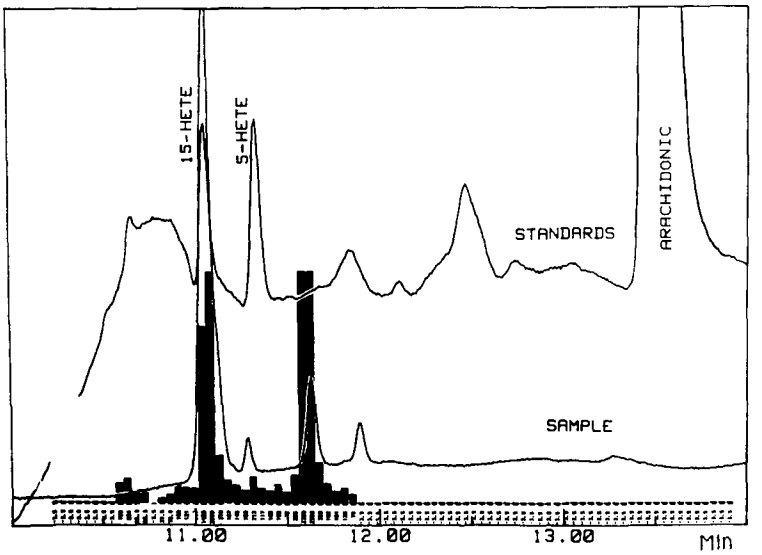

Fig. 5. Scans of radioautographs superimposed on the UV chromatogram of HETE metabolites of platelets of arachidonic acid. Conditions as in Fig. 2.

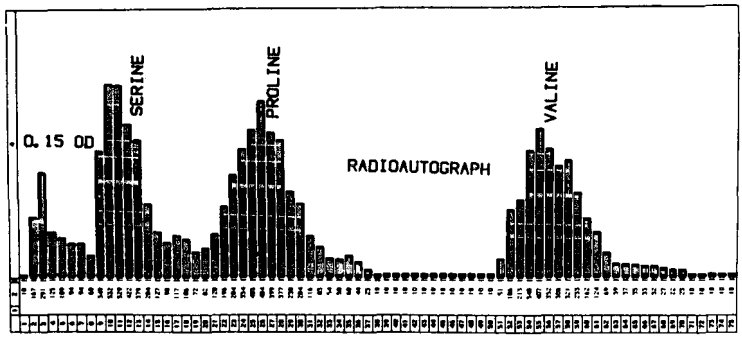

Fig. 6. Standard curves of scans of 10–120 dpm each of [^{3}H]thromboxane and [^{14}C]arachidonic acid.

proportional counter was similar. Here again, it was likely that the automatic scanner was not always directly over the radioactive spots during the counting intervals. With 5 min over each row of spots (rows were scanned, rather than columns, because of the size of the paper and the scanner), similar records were obtained.

DISCUSSION

It was apparent that the mechanical capabilities of the two scanners were not being fully utilized in scanning the evenly spaced, small, well-separated spots. Appreciably greater sensitivity was obviously available by coordinating the separation of the spots with the counter spacing or the settings of the position of the proportional counter.

All these procedures involved simultaneous radioassay by an array of detectors, which, for this purpose, includes both the position sensitive proportional counter and the photographic film, as well as the Geiger counter array. Upon noting the high sensitivity of off-line detection with this approach, we designed a method for extending it to achieving more sensitive on-line radiochromatography with approximately the same convenience as flow-through detection. One possible embodiment, employing a Geiger counter array, is as follows: fractions of the column effluent are deposited in pockets formed on a long strip of fluorocarbon film that are moved step-wise past the column exit. At the first, second and third steps in the movement of the film, the solvents are evaporated to dryness. The pockets containing the condensed radioactivity then travel past the array of detectors so that each spot is counted by each detector sequentially. The counts recorded by each detector during each time interval are stored in memory. The counting rates are summed continuously by a formula that takes into account the position of each sample passing through the array: during the third counting interval, for example, the output of the third detector is summed in the memory bank corresponding to the first sample, etc. This approach gives defined resolution, the same resolution as flow-through detection, but each sample is counted many times as long, (*e.g.* ten times with the ten-detector array) with corresponding increases in sensitivity and precision.

It is contemplated that either one of the gas detector systems described here could be used. The same approach could also conceivably be used with internal liquid

or solid scintillation counting, using a similar succession or array of detectors. For example, liquid scintillation fluid can be added to the HPLC column effluent and the combined stream then led to a long length of PTFE tubing. Baba and co-workers[4,5] described then delivering a stream of this kind through five scintillation counters in series, achieving the predicted increase in precision from summing the radioactivity. They also noted, however, that there was an appreciable increase in peak width from the first to the fifth detector, caused presumably by mixing of one part of the effluent with the next. This effect is particularly troublesome with detergent-containing scintillation fluids which tend to wet even PTFE tubing. Mixing can be reduced somewhat by segmenting the stream into distinct fractions with an immiscible liquid. Each segment could then be passed stepwise, every 15 s for example, through a series of light detectors where the scintillations of each during each counting interval are summed as described above.

Liquid scintillation counting generally offers higher sensitivity, particularly for ^{3}H, than external counting with a gas detector, because of reduced self absorption of the β-particles. However, since photomultiplier detectors are currently much more expensive, the cost of an array of pairs of light detectors, arranged for coincidence detection, or a scintillation camera such as is used nuclear medicine, would be comparatively high.

The increase in sensitivity achievable by passing 15- or 20-s portions of the column effluent through a succession of detectors can be estimated from the increased time of counting: the counts recorded in ten detectors, if a fraction spends 15–20 s in front of each, would predictably be ten times the number recorded if the sample passes in front of a single detector of the same kind for the same time period. On the other hand, if the sensitivity of an array of ten external gas detectors is compared with that of a single flow-through scintillation counter, that accepts the same size fraction of the effluent (*e.g.* a 15-s portion) a smaller improvement would be predicted. From consideration of geometry alone, and neglecting self-absorption of the β-particles in the sample or the carrier, the efficiency of external gas detectors reaches a maximum of only 50%, compared to *ca.* 80% in internal liquid scintillation systems. A counter placed above the sample does not "see" the β-particles that are directed downward. Some improvement might be obtained by employing opposing detectors, one on each side of the carrier strip, arranging the spacing so that electric discharges in one do not affect the other. It would probably be more straightforward to use additional detectors to increase the number of steps in the array, increasing the sensitivity in direct proportion to their number, even though this would increase the time of completion of the assay.

Even though the procedure described involves fractionating the effluent, it accomplishes the primary objective of on-line detection: obtaining the result at close to the same time each portion of the effluent leaves the column. With the system described here, the counts in each memory bank can be portrayed continuously. The counts in a fraction can be examined within a very short interval after it leaves the column. However, additional data continues to accumulate at succeeding times, increasing the precision of the assay: as the tenth portion in a sequence enters the detection array, counts are still being recorded from the first nine, etc. The assay is complete only a few minutes after the analysis. In this regard, the radioassay is as on-line as detection that uses a post-column reaction that requires several minutes for completion. Unlike other

forms of on-line detection, the fractions remain available for repeated analysis off-line, when even greater sensitivity is needed.

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SIMULTANEOUS GAS CHROMATOGRAPHY OF VOLATILE AND NON-VOLATILE CARBOXYLIC ACIDS AS *tert.*-BUTYLDIMETHYLSILYL DERIVATIVES

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SUMMARY

Solid phase extraction with subsequent ion pair formation and silylation was investigated for the simultaneous trace enrichment of volatile and non-volatile carboxylic acids from complex aqueous samples. The solid phase extraction of acids was performed using Chromosorb P as the solid sorbent. The ether eluate was treated with triethylamine. The resulting triethylammonium salts of the acids were converted to stable *tert.*-butyldimethylsilyl (TBDMS) derivatives, which were analyzed by gas chromatography (GC) and GC–mass spectrometry. The characteristic M – 57 ions in the mass spectra of TBDMS derivatives enabled rapid identification of acids. The application of the method to the organic acid profiling of urine and saliva samples is demonstrated.

INTRODUCTION

The organic acids, a group of the most widely occurring compounds in biological samples, are important indicators of a wide variety of biological, physiological and fermentation processes[1–6]. The organic acid fraction of complex biological samples contains a wide range of structural types of acids, including short-chain volatile fatty acids, long-chain non-volatile fatty acids, mono-, and polycarboxylic acids with hydroxy or keto functional groups of both aliphatic and aromatic character. A number of gas chromatographic (GC) methods have been developed to separate one or several classes of these acids. Simultaneous analysis of short-chain volatile fatty acids and various non-volatile carboxylic acids is a commonly encountered problem in the organic acid profiling studies.

Prior to analysis, the organic acids are isolated from complex sample matrices,

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mainly by either solvent extraction[2] or anion-exchange method[6]. In modern analysis, conventional liquid–liquid partitioning extraction is being replaced by liquid–solid adsorption, *i.e.* solid phase extraction (SPE), where compounds of interest are enriched on suitable sorbents, followed by elution with organic solvents. The advantages of SPE methods are well established. Non-polar graphitized carbon black[1], C_{18} bonded silica and XAD-4[4] have been used to enrich organic acids. The anion-exchange approach, being more laborious than solvent extraction procedures, has been reported to have two major drawbacks: the inorganic acid interference and the loss of the more volatile acids[3]. Previously, we reported an efficient two-step SPE method with Chromosorb P as the solid sorbent and diethyl ether as the eluent for enrichment of volatile fatty acids (C_2–C_5) from fermentation media and body fluids[4].

Generally, the carboxyl groups of organic acids are converted to either the alkyl esters (methyl or butyl) or trimethylsilyl esters prior to GC analysis[2–5]. Recently, separation of Krebs cycle and related acids as *tert.*-butyldimethylsilyl (TBDMS) esters and ethers was reported. TBDMS ether derivatives of hydroxyl groups have been widely used, principally because of their high hydrolytic stability and superior GC and mass spectrometric (MS) properties[6], while TBDMS esterification of carboxyl groups has received little attention.

In a previous report[7], we described the GC analysis of volatile fatty acids (C_1–C_7) as their TBDMS esters: the acids were converted to non-volatile triethylammonium (TEA) salts to minimize the loss due to the evaporation during the sample work-up, followed by reaction of the salts with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) to afford TBDMS esters.

The present work was undertaken to examine the SPE method[4,8] and the direct TBDMS derivatization of TEA salts[7] for the simultaneous determination of various volatile and non-volatile carboxylic acids in aqueous samples. The authenticity of the TBDMS derivatives was verified by GC–MS.

EXPERIMENTAL

Materials

Twenty-two saturated fatty acids (C_1–C_{20}) were obtained from Analabs (New Haven, CT, U.S.A.) and Alltech (Deerfield, IL, U.S.A.). The following thirteen aliphatic and sixteen aromatic multifunctional acids were obtained from various commercial vendors such as Sigma (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.): lactic, glycolic, oxalic, malonic, succinic, methylsuccinic, fumaric, adipic, 3-methyladipic, malic, suberic, tartaric, citric, benzoic, phenylacetic, *trans*-cinnamic, 5-phenylvaleric, *p*-aminobenzoic, mandelic, phenyllactic, hippuric, γ-hydroxybenzoic, vanillic, syringic, α-resorcylic, *p*-hydroxymandelic, γ-resorcylic, homogentisic, and protocatechuic acids. TEA was purchased from Aldrich, diethyl ether from Tedia (Fairfield, OH, U.S.A.), MTBSTFA from Pierce (Rockford, IL, U.S.A.), dichloromethane and isooctane from Burdick & Jackson (Muskegon, MI, U.S.A.), methanol from Shinyo (Kyoto, Japan), sodium chloride and sodium bicarbonate from Ishizu (Osaka, Japan), sulfuric acid (98.08%) from Junsei (Tokyo, Japan), and *n*-hydrocarbon standards (C_8–C_{28}) from Polyscience (Niles, IL, U.S.A.). All solvents and reagents were of analytical grade and were used as received, except TEA, which was distilled over potassium hydroxide. Chromosorb P (acid-washed, 80–100 mesh) was

obtained from Supelco (Bellefonte, PA, U.S.A.). A U-shaped glass column (6 mm I.D.) was packed with Chromosorb P (2.3 g), washed successively with methanol, dichloromethane, and diethyl ether, and activated at 200°C overnight prior to being used as a solid phase extraction column.

Acid solutions

Four acid stock solutions and one internal standard solution were prepared for this study as follows: volatile fatty acid mixture (C_1–C_7, 10 μg each per μl in diethyl ether), fatty acid mixture (C_8–C_{20} 10 μg each per μl in dichloromethane), aliphatic multifunctional carboxylic acid mixture (thirteen acids, 50 μg each per μl in methanol), aromatic carboxylic acid (fifteen acids, 50 μg each per μl in methanol) and *trans*-cinnamic acid solution as an internal standard solution (100 μg per μl in methanol).

tert.-Butyldimethylsilylation

Appropriate amounts of carboxylic acid mixtures were diluted to 1.5 ml with diethyl ether, containing 100 μg of *trans*-cinnamic acid as an internal standard. The ether solution was then mixed with 20 μl of triethylamine in a Reacti Vial (Pierce). The ether was reduced to *ca.* 50 μl under a gentle stream of dry nitrogen at room temperature. To the vial were added 20 μl of MTBSTFA and 60 μl of isooctane, and the vial was tightly closed with a PTFE-lined screw cap. The mixture was subjected to GC analysis either directly, or after it was heated at 60°C for 30 min or up to 8 h.

To test the effect of TEA treatment on the recoveries of volatile fatty acids (C_1–C_7), untreated samples were prepared in the same manner except for the addition of TEA.

The samples for calibration were prepared by allowing increasing amounts of acids containing 100 μg of the internal standard to react directly with TEA (20 μl), and MTBSTFA (20 μl) in the presence of isooctane (60 μl) in reacti vials.

Aliquots of the reaction mixtures were examined directly by GC–MS.

Solid phase extraction

To 1 ml of aqueous samples or biological samples 100 μg/ml of the internal standard was added and the solution was made basic by saturating with solid sodium bicarbonate. After extraction with diethyl ether, the ether phase was discarded and the aqueous phase was subjected to SPE, as described in ref. 4. Briefly, following the acidification with concentrated sulfuric acid (0.1 ml) and saturation with sodium chloride (400 mg), the aqueous phase was loaded onto a Chromosorb P column under nitrogen pressure. The packing was wetted up to 80% while the remaining 20% was dry. The organic acids were then eluted from the Chromosorb P with diethyl ether, and the first 1.5 ml of ether eluate was collected in a Reacti Vial containing 20 μl TEA, followed by the evaporation and derivatization, as described above.

Gas chromatography

GC analyses were conducted with a Shimadzu GC-9A gas chromatograph, equipped with a flame ionization detector and interfaced with a Shimadzu C-R2AX data processor, which provided peak area and retention time date (Shimadzu, Kyoto, Japan). A DB-1 (J&W Scientific, Rancho Cordova, CA, U.S.A.) fused-silica capillary

column (30 m × 0.32 mm I.D., 0.25 μm particle size) was used for this study. Nitrogen at a flow-rate of 0.9 ml/min was used as the carrier gas, and 0.8-μl aliquots of samples were injected with a split ratio of 15:1. After an initial hold time of 2 min at 60°C, the oven temperature was programmed to 280°C at a rate of 4°C/min. The injector and detector temperatures were maintained at 300°C. A standard solution of *n*-hydrocarbons (C_8–C_{28}) in isooctane was simultaneously injected with the samples to allow conversion of retention times to retention indices. Samples were analyzed in triplicate.

Gas chromatography–mass spectrometry

A Hewlett Packard (Avondale, PA, U.S.A.) HP 5890 A gas chromatograph, interfaced to an HP 5970 MSD 70-eV electron-impact mode, which was on-line to an HP 35741 Chemstation data system, was used with an HP-1 cross-linked capillary column (16 m × 0.20 mm I.D., 0.33 μm particle size) to obtain mass spectra. Samples were introduced in the split-injection mode (10:1) at 270°C, and the oven temperature was initially 100°C for 2 min, then programmed to 300°C at a rate of 15°C/min. The interface and ion source temperatures were 300°C and 200°C, respectively. The scan-rate of the mass scanning from 50 to 600 a.m.u. was 0.78 scan per s.

RESULTS AND DISCUSSION

In a previous report[8], we established that TEA salts of volatile fatty acids can be converted to their TBDMS esters directly after reaction with MTBSTFA in isooctane. An excess of TEA (20 μl) was used, and the removal of diethyl ether under a stream of nitrogen was stopped when TEA started to evaporate, in order to obtain high yields of TEA salts. The effect of TEA treatment on the recoveries of volatile fatty acids, diluted in ether solution, was evaluated. As shown in Table I, the TEA treatment minimizes the loss of acids and improves the precision of the overall derivatization procedure.

TABLE I

EFFECT OF TRIETHYLAMINE TREATMENT ON THE RECOVERIES OF VOLATILE FATTY ACIDS

A 1.5-ml volume of diethyl ether, containing 70 μg each of C_1 to C_7 volatile fatty acids and 100 μg of *tert.*-cinnamic acid, was subjected to silylation as described in the text (n = 3). C.V. = Coefficient of variation.

Acid	*With TEA treatment*		*Without TEA treatment*	
	Recovery (%)	*C.V. (%)*	*Recovery (%)*	*C.V. (%)*
Formic	91.6	0.1	Trace	–
Acetic	99.8	0.2	Trace	–
Propionic	97.2	<0.1	Trace	–
Isobutyric	100.0	<0.1	2.8	34.5
Butyric	100.0	<0.1	5.6	25.0
Isovaleric	100.0	0.3	8.4	20.7
Valeric	100.0	0.9	20.4	4.9
Caproic	100.0	1.2	44.5	6.4
Enanthic	100.0	0.2	56.0	3.6

The TBDMS esterification of non-sterically hindered carboxyl groups was complete when their TEA salts were mixed with MTBSTFA at room temperature. Even the highly hindered hydroxyl and carboxyl groups of tartaric acid and γ-resorcylic acid as their TEA salts were quantitatively silylated by heating at 60°C for 4 h and 8 h, respectively. However, with *p*-aminobenzoic acid and hippuric acid, N-silylation did not take place, even with excess MTBSTFA for extended periods.

The derivative yields of the carboxylic acids studied were quantitative. The calibration curves were linear, with correlation coefficients (r^2) varying from 0.9944 to 0.9999, over the range 10–80 μg for the fatty acids, and 50–400 μg for the aliphatic and aromatic multifunctional carboxylic acids under the present condition. The quantitative yields of TBDMS derivatives are exemplified by the calibration plots of formic, capric, tridecanoic, arachidic, oxalic, lactic, malic, tartaric, citric, benzoic, *p*-aminobenzoic, phenylacetic, mandelic, and phenyllactic acids in Figs. 1–3.

With the present derivatization procedure, a single chromatographic peak with almost no tailing is obtained for each of the carboxylic acids studied, as illustrated in Fig. 4. Volatile formic, acetic, and propionic acids are well separated from the solvent and reagent peaks. Mandelic acid is eluted with lauric acid, 3-methyladipic acid with tridecanoic acid, and α-resorcylic acid with margaric acid. For their complete separation a more polar phase appears to be needed.

TBDMS derivatives of the carboxylic acids investigated were found to be stable for at least six months when stored at 4°C. The present direct silylation of the TEA salts with MTBSTFA has the following advantages: (1) TEA salts are non-volatile, and this minimizes the loss of volatile fatty acids during the sample work-up; (2) both TEA and TEA salts are soluble in the mixture of MTBSTFA and isooctane; (3) the acids are easily released from TEA salts and silylated quantitatively during the reaction; (4)

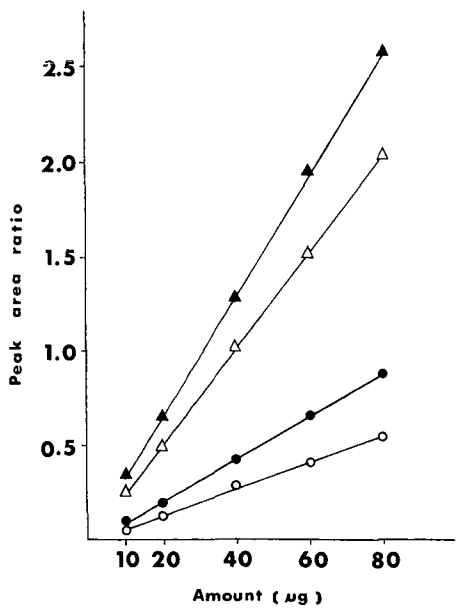

Fig. 1. Calibration curves for formic (@), capric (⅛), tridecanoic (△) and arachidic (⅓) acids.

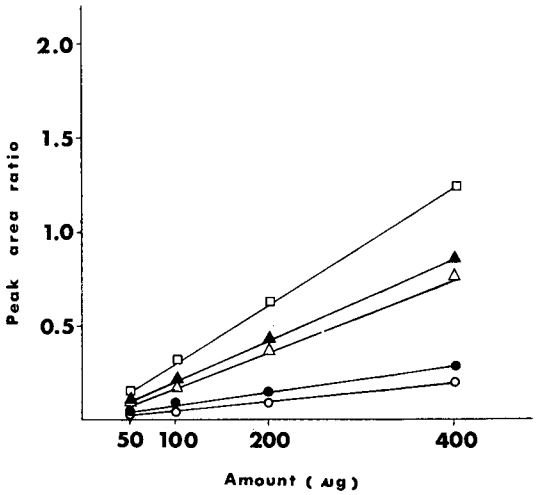

Fig. 2. Calibration curves for oxalic (@), lactic (⅛), malic (△), tartaric (⅓) and citric (□) acids.

aprotic TEA does not undergo silylation; (5) without further processing, the reaction mixtures can be examined directly by GC and GC–MS; (6) TEA is eluted with isooctane, and thus does not interfere with the analysis of volatile acids; (7) hydroxyl and carboxyl functions are quantitatively converted to TBDMS ethers and esters; (8)

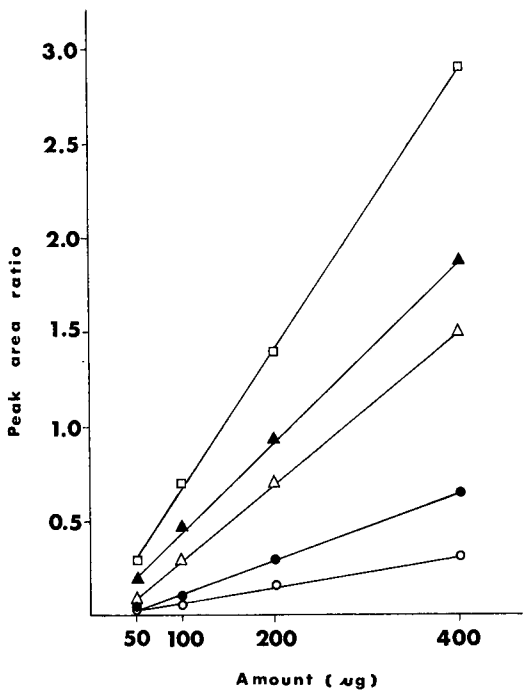

Fig. 3. Calibration curves for benzoic (@), *p*-aminobenzoic (⅛), phenylacetic (△), mandelic (⅓) and phenyllactic (□) acids.

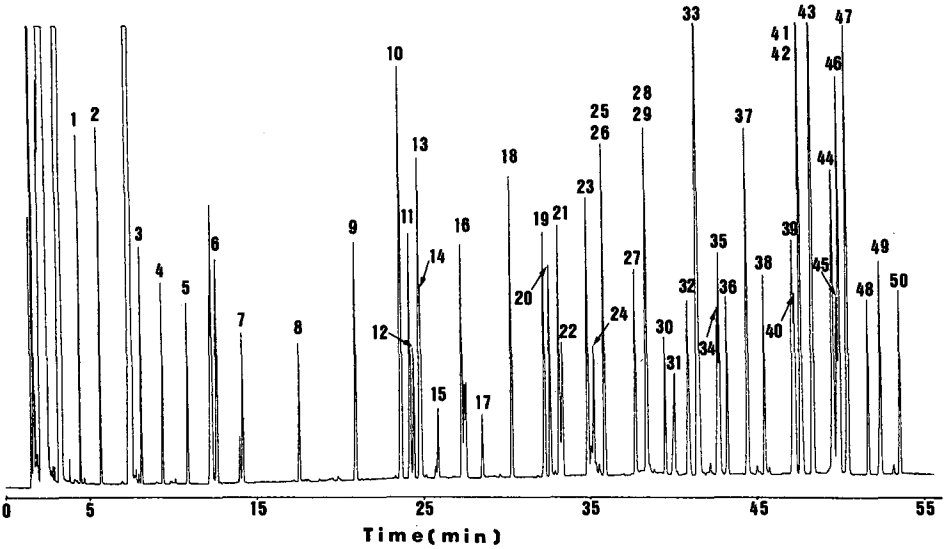

Fig. 4. Chromatogram of a mixture of carboxylic acids as the *tert.*-butyldimethylsilyl derivatives. GC conditions: DB-1 fused-silica capillary column (30 m × 0.32 mm I.D., 0.25 μm), initially at 60°C for 2 min, then programmed to 280°C at 4°C/min; 0.8 μl sample, injected with split ratio of 15:1; both injector and detector temperatures at 300°C; nitrogen as the carrier gas at 0.9 ml/min. Peaks: 1 = formic, 2 = acetic, 3 = propionic, 4 = isobutyric, 5 = butyric, 6 = isovaleric, 7 = valeric, 8 = caproic, 9 = enanthic, 10 = benzoic, 11 = caprylic, 12 = lactic, 13 = phenylacetic, 14 = glycolic, 15 = oxalic, 16 = pelargonic, 17 = malonic, 18 = capric, 19 = succinic, 20 = methylsuccinic, 21 = undecanoic, 22 = fumaric, 23 = 5-phenylvaleric, 24 = *p*-aminobenzoic, 25 = lauric, 26 = mandelic, 27 = adipic, 28 = 3-methyladipic, 29 = tridecanoic, 30 = phenyllactic, 31 = hippuric, 32 = myristic, 33 = *p*-hydroxybenzoic, 34 = malic, 35 = suberic, 36 = pentadecanoic, 37 = vanillic, 38 = palmitic, 39 = syringic, 40 = tartaric, 41 = margaric, 42 = α-resorcylic, 43 = *p*-hydroxymandelic, 44 = γ-resorcylic, 45 = stearic, 46 = homogentisic, 47 = protocatechuic, 48 = nonadecanoic, 49 = citric, 50 = arachidic acid.

amino and amide functions are not capable of being silylated under the present reaction condition.

All the TBDMS derivatives were subjected to GC–MS analysis. The retention index and electron-impact MS data are summarized in Table II. As is characteristic of TBDMS derivatives, the molecular ions and M − 15 ions are absent or of low intensity, but the M − 57 ions, due to the loss of the *tert.*-butyl group function from the molecular ions are very intense, thus enabling rapid identification of acids.

For most of the monocarboxylic acids, M − 57 ions constitute the base peaks, and the ion of high intensity at $m/z = 75$ corresponds to $HOSi(CH_3)_2$, as seen in Fig. 5. The mass spectra of the TBDMS derivatives of lactic, malic, citric, fumaric, and succinic acids do not agree well with those recorded at an electron energy of 22.5 eV. As in the spectra of trimethylsilyl derivatives, the ion at $m/z = 73$, corresponding to $Si(CH_3)_3$, is the base peak for the bisTBDMS derivatives of acids with vicinal hydroxyl groups, two neighboring hydroxyl and carboxyl groups, or dicarboxylic acids, as shown in Fig. 6. Other prominent ions at $m/z = 115$, 157, and 189 correspond to $Si(CH_3)_2C(CH_3)_3$, $(CH_3)_3SiOSi(CH_3)_2$, and $(CH_3)_3C(CH_3)_2$ $SiOSi(CH_3)_2$, respectively.

TABLE II

GAS CHROMATIGRAPHIC AND MASS SPECTRAL DATA OF TBDMS DERIVATIVES OF CARBOXYLIC ACIDS

Retention index (RI) values on a DB-1 capillary column (30 m × 0.32 mm I.D., 0.25-μm) at 60°C (2 min) to 280°C at 4°C/min, and the relative abundance of ions are in parentheses.

Acid	*RI*	*Mol.wt.*	$(M-57)^+$	*Major ions*				
Formic	858	160	103(100)	75(56)	73(7)	59(5)	57(4)	61(4)
Acetic	916	174	117(91)	75(100)	73(5)	59(3)	57(3)	60(3)
Propionic	990	188	131(80)	75(100)	73(12)	57(8)	59(4)	58(3)
Isobutyric	1029	202	145(82)	75(100)	73(20)	115(7)	59(4)	57(4)
Butyric	1074	202	145(73)	75(100)	73(13)	115(4)	59(4)	57(3)
Isovaleric	1126	216	159(73)	75(100)	73(15)	57(6)	115(4)	59(4)
Valeric	1174	216	159(72)	75(100)	73(13)	57(6)	115(4)	59(4)
Caproic	1283	230	173(83)	75(100)	73(17)	131(13)	115(5)	59(5)
Enanthic	1378	244	187(76)	75(100)	73(17)	131(14)	129(5)	117(5)
Caprylic	1465	258	201(100)	75(94)	131(15)	129(10)	117(7)	57(7)
Pelargonic	1574	272	215(97)	75(100)	73(22)	131(16)	129(16)	117(8)
Capric	1674	286	229(100)	75(73)	73(19)	131(14)	129(14)	55(7)
Undecanoic	1773	300	243(100)	75(78)	73(17)	129(16)	131(12)	55(7)
Lauric	1874	314	257(100)	75(59)	73(15)	129(14)	131(10)	117(8)
Tridecanoic	1973	328	271(100)	75(59)	73(14)	129(14)	131(8)	55(6)
Myristic	2075	342	285(100)	75(45)	129(13)	73(12)	131(8)	55(6)
Pentadecanoic	2174	356	299(100)	75(45)	129(14)	73(13)	117(9)	131(7)
Palmitic	2275	370	313(100)	75(38)	129(13)	73(10)	117(8)	131(6)
Margaric	2373	384	327(100)	75(43)	129(15)	73(13)	117(8)	131(6)
Stearic	2476	398	341(100)	75(28)	129(10)	73(8)	117(6)	131(4)
Nonadecanoic	2576	412	355(100)	75(36)	129(12)	73(12)	117(8)	131(5)
Arachidic	2677	426	369(100)	75(19)	129(7)	73(7)	117(6)	131(3)
Lactic	1483	318	261(24)	73(100)	147(86)	189(25)	133(21)	75(20)
Glycolic	1499	304	247(24)	73(100)	147(51)	189(32)	75(13)	133(12)
Oxalic	1535	318	261(13)	73(100)	147(32)	75(10)	59(9)	133(6)
Malonic	1604	332	275(30)	73(100)	147(27)	75(23)	189(13)	133(12)
Succinic	1743	346	289(51)	73(100)	147(48)	75(36)	133(9)	116(9)
Methylsuccinic	1755	360	303(58)	73(100)	147(60)	75(36)	59(9)	123(8)
Fumaric	1779	344	287(100)	73(48)	75(21)	57(11)	133(8)	84(7)
Adipic	1948	374	317(71)	73(100)	75(79)	111(35)	141(18)	55(15)
3-Methyladipic	1973	388	331(68)	73(100)	75(80)	125(36)	155(17)	69(13)
Malic	2151	476	419(22)	73(100)	147(23)	287(20)	115(15)	75(13)
Suberic	2157	402	345(95)	73(100)	75(89)	129(19)	169(14)	55(13)
Tartaric	2355	606	549(27)	73(100)	147(19)	115(9)	75(7)	417(7)
Citric	2617	648	591(25)	73(100)	459(35)	147(19)	431(14)	357(11)
Benzoic	1460	236	179(100)	105(58)	77(46)	135(30)	51(8)	75(5)
Phenylacetic	1494	250	193(91)	75(100)	91(22)	73(22)	65(9)	137(8)
trans-Cinnamic	1752	262	205(100)	131(49)	103(42)	75(39)	161(32)	77(28)
5-Phenylvaleric	1836	292	235(97)	75(100)	91(29)	73(19)	131(16)	117(15)
p-Aminobenzoic	1850	251	194(90)	120(100)	150(62)	65(30)	92(29)	75(16)
Mandelic	1875	380	323(26)	73(100)	147(39)	221(32)	295(32)	75(14)
Phenyllactic	2016	394	337(36)	73(100)	147(88)	309(27)	75(22)	133(16)
Hippuric	2035	293	236(53)	105(100)	77(31)	73(21)	75(20)	192(10)
p-Hydroxybenzoic	2097	366	309(100)	73(45)	265(33)	235(19)	57(11)	135(8)
Vanillic	2228	396	339(100)	267(42)	73(24)	126(15)	193(12)	295(12)
Syringic	2354	426	369(100)	297(40)	73(20)	149(13)	141(13)	223(9)
α-Resorcylic	2371	496	439(100)	73(70)	267(10)	309(9)	57(9)	147(5)
p-Hydroxymandelic	2400	510	453(29)	73(100)	351(92)	425(45)	147(22)	75(11)
γ-Resorcylic	2467	496	439(100)	73(40)	57(8)	170(5)	397(5)	147(4)
Homogentisic	2486	510	453(43)	73(100)	75(16)	147(14)	327(13)	57(8)
Protocatechuic	2511	496	439(100)	73(61)	193(12)	395(7)	223(5)	267(5)

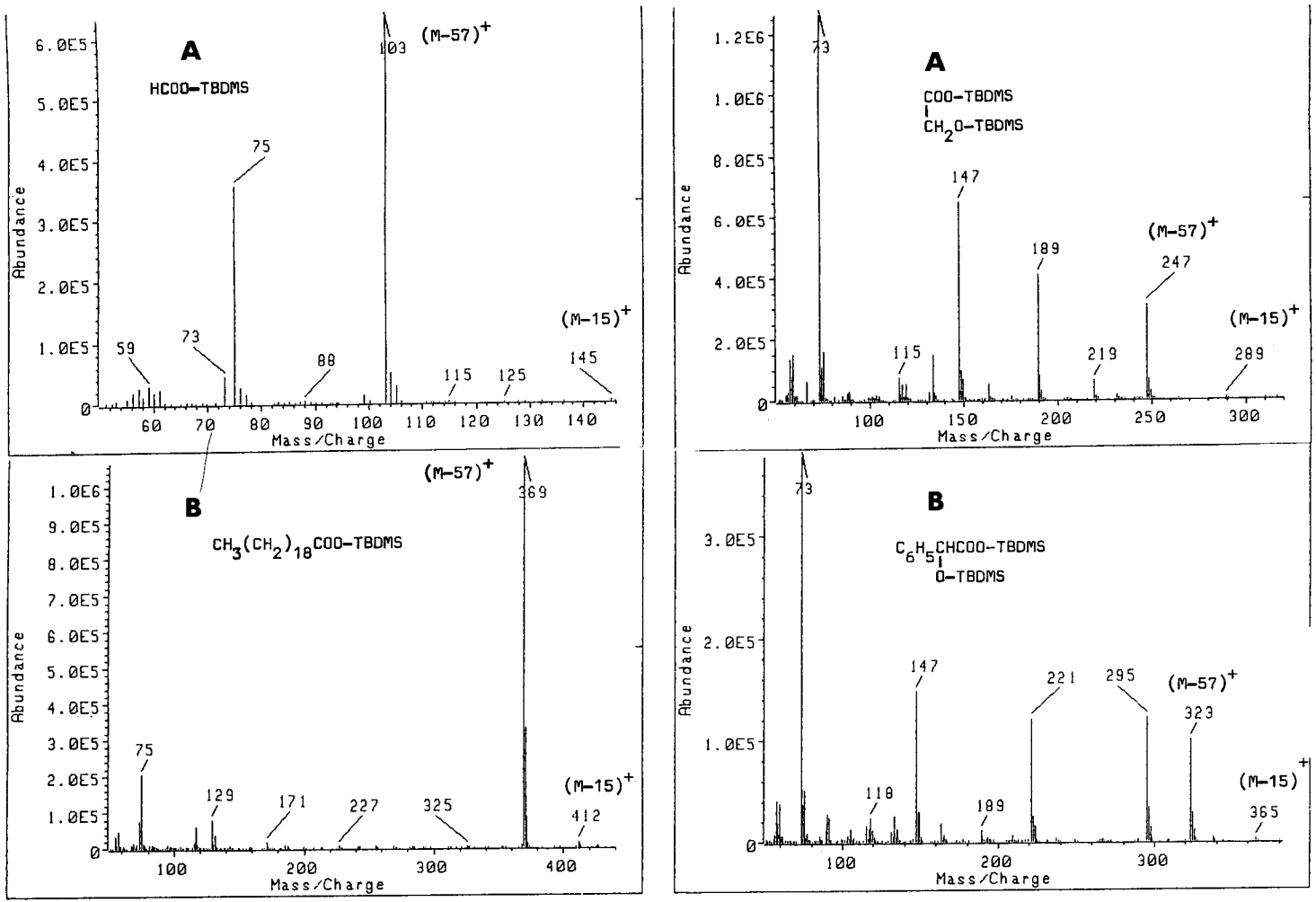

Fig. 5. Electron-impact mass spectra of TBDMS derivatives of formic acid (A) and arachidic acid (B).

Fig. 6. Electron-impact mass spectra of TBDMS derivatives of glycolic acid (A) and mandelic acid (B).

TABLE III

OVERALL EFFICIENCY OF THE COMBINED SOLID PHASE EXTRACTION AND DIRECT SILYLATION OF THE TRIETHYLAMMONIUM SALTS OF FATTY ACIDS

Fatty acid mixture (40 μg each) was added to 1 ml of water, containing 100 μg of *trans*-cinnamic acid and subjected to solid phase extraction and silylation, as described in the text ($n = 3$).

Acid	*Recovery (%)*	*C.V. (%)*	*Acid*	*Recovery (%)*	*C.V. (%)*
Formic	30.8	0.3	Capric	98.0	0.3
Acetic	92.5	1.0	Lauric	96.4	0.7
Propionic	90.1	<0.1	Tridecanoic	98.7	0.1
Isobutyric	88.6	<0.1	Myristic	99.6	0.2
Butyric	93.6	<0.1	Pentadecanoic	98.1	0.2
Isovaleric	92.8	0.6	Palmitic	100.0	0.4
Valeric	90.8	1.1	Margaric	100.0	0.6
Caproic	92.1	1.5	Stearic	100.0	0.6
Enanthic	95.7	1.9	Nonadecanoic	100.0	0.6
Caprylic	98.6	0.2	Arachidic	100.0	0.5
Pelargonic	100.0	<0.1			

TABLE IV

OVERALL EFFICIENCY OF THE COMBINED SOLID PHASE EXTRACTION AND DIRECT SILYLATION OF THE TRIETHYLAMMONIUM SALTS OF ALIPHATIC MULTIFUNCTIONAL ACIDS

Aliphatic acid mixture (100 μg each) was added to 1 ml of water, containing 100 μg of *trans*-cinnamic acid and subjected to solid phase extraction and silylation, as described in the text ($n = 3$).

Acid	*Recovery (%)*	*C.V. (%)*	*Acid*	*Recovery (%)*	*C.V. (%)*
Lactic	81.7	0.4	Adipic	84.4	0.6
Glycolic	88.1	0.6	3-Methyladipic	99.2	0.2
Oxalic	81.4	0.4	Malic	82.0	1.2
Malonic	100.0	0.2	Suberic	91.0	0.1
Succinic	100.0	0.2	Tartaric	81.7	0.9
Methylsuccinic	100.0	0.1	Citric	81.1	0.4
Fumaric	100.0	0.2			

TABLE V

OVERALL EFFICIENCY OF THE COMBINED SOLID PHASE EXTRACTION AND DIRECT SILYLATION OF THE TRIETHYLAMMONIUM SALTS OF AROMATIC MULTIFUNCTIONAL ACIDS

Aromatic acid mixture (100 μg each) was added to 1 ml of water, containing 100 μg of *trans*-cinnamic acid and subjected to solid phase extraction and silylation, as described in the text ($n = 3$).

Acid	*Recovery (%)*	*C.V. (%)*	*Acid*	*Recovery (%)*	*C.V. (%)*
Benzoic	100.0	0.2	Vanillic	99.8	0.3
Phenylacetic	95.9	0.4	Syringic	100.0	0.6
5-Phenylvaleric	99.6	0.1	α-Resorcylic	100.0	0.3
p-Aminobenzoic	99.4	0.2	*p*-Hydroxymandelic	99.5	0.3
Mandelic	99.0	1.0	γ-Resorcylic	99.9	0.5
Phenyllactic	99.4	0.2	Homogentisic	100.0	0.3
Hippuric	100.0	1.5	Protocatechuic	99.6	1.0
p-Hydroxybenzoic	100.0	1.2			

In spite of recent advances in GC, the simultaneous detection of volatile short-chain fatty acids as well as various non-volatile carboxylic acids is still a difficult task. For this purpose, we combined the SPE method, developed for the trace enrichment of organic acids[4], and the direct TBDMS derivatization of their TEA salts[7]. Prior to SPE, aqueous samples were treated with sodium bicarbonate, followed by extraction with diethyl ether to remove the interfering non-carboxylic compounds, such as neutral, basic, and phenolic compounds. The resulting aqueous phase was acidified and loaded onto a Chromosorb P column. Free carboxylic acids were then eluted with diethyl ether, while water, inorganic acids and salts, urea, and other water soluble, polar compounds were held up by the hydrophilic Chromosorb P. Eluted acids were converted to TEA salts, which were then concentrated and directly silylated to TBDMS derivatives.

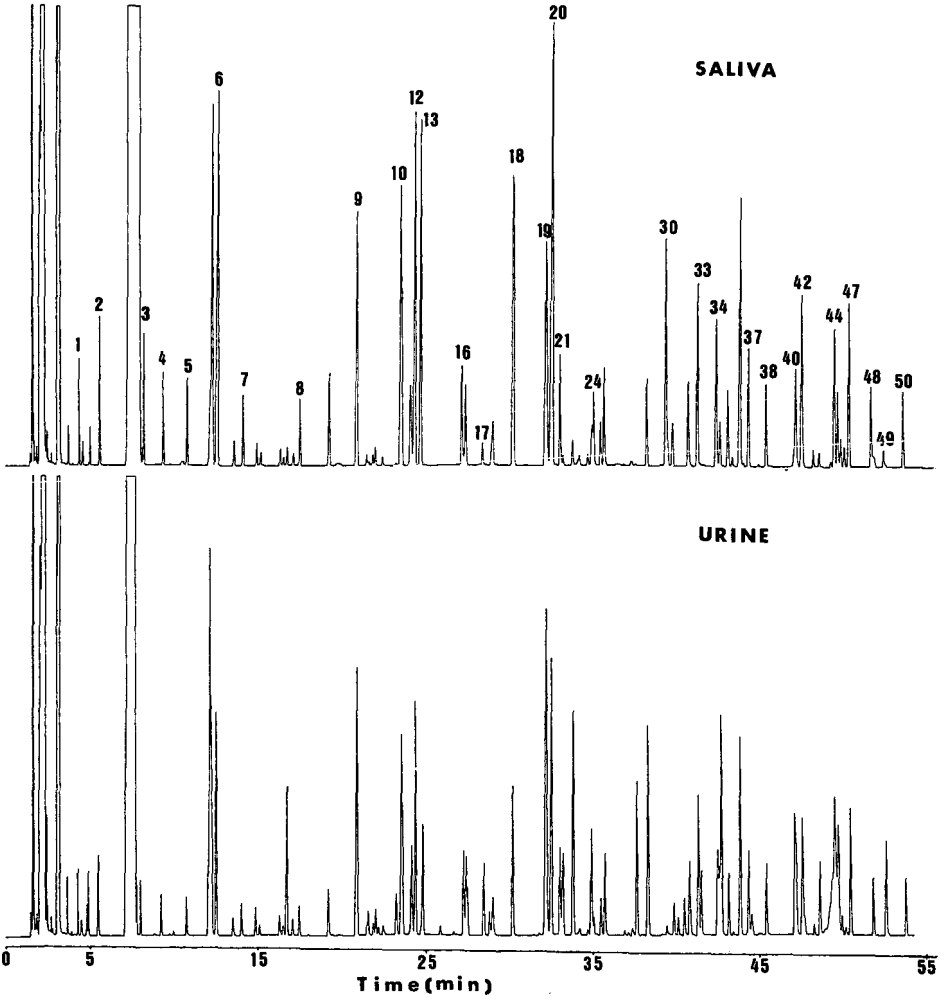

Fig. 7. Chromatograms of organic acids in saliva and urine from a normal person, A. A 1-ml volume of each sample was subjected to combined SPE and direct silylation, as described in the text. GC conditions and peak identities as in Fig. 4.

The recoveries of the three classes of acids from water samples, enriched with the acids at known concentrations, are listed in Tables III, IV, and V, respectively. Most of the acids could be recovered in excellent yields with good precision, except for formic acid and some polar hydroxy carboxylic acids. The exceptionally low recovery rate of formic acid is probably due to its higher affinity for the hydrophilic Chromosorb P.

When applied to body fluids, the present method demonstrated very efficient and highly specific sampling of carboxylic acids from complex aqueous mixtures. More than 40 acids were detected and tentatively identified, both in urine and saliva samples from two normal individuals, as shown in Figs. 7 and 8. Common interfering compounds in biological samples, such as urea, phenols, and phosphate were effectively excluded by the present procedure.

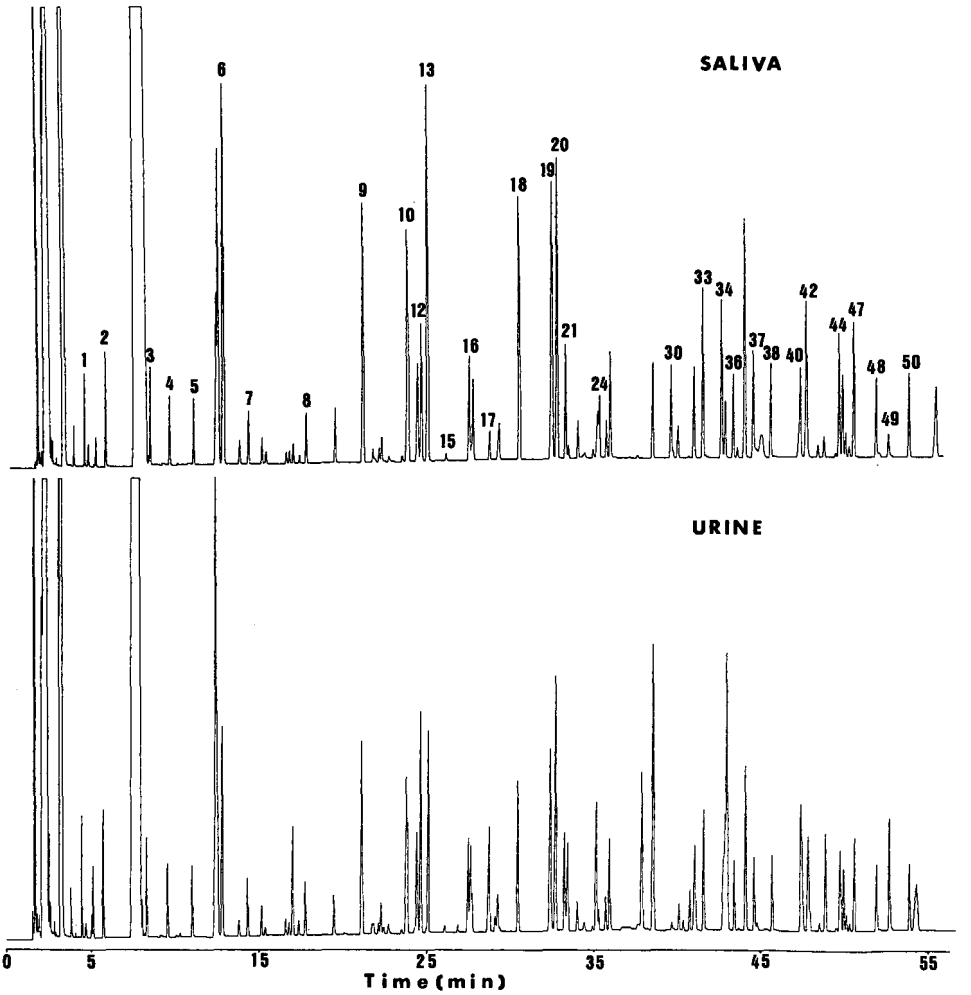

Fig. 8. Chromatograms of organic acids in saliva and urine from a normal person, B. A 1-ml volume of each sample was subjected to combined SPE and direct silylation, as described in the text. GC conditions and peak identities as in Fig. 4.

In conclusion, it can be stated that the present method is simple, rapid and specific as the method of choice for the simultaneous detection of various classes of carboxylic acids in complex sample matrices.

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IMPROVED FLAME PHOTOMETRIC DETECTOR FOR THE ANALYSIS OF SULFUR COMPOUNDS BY GAS CHROMATOGRAPHY

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SUMMARY

The design of the flame photometric detector has not been changed significantly in more than two decades. Major problems in the use of this detector for quantitative analysis are variation in the response with molecular structure of a sulfur-containing species, and hydrocarbon quenching. The variable response noted with the FPD may, in part, be associated with the wavelength shift of the interference filter bandpass for off-axis radiation.

We have developed a flame photometric detector which employs rare-earth glass filters that transmit a wavelength-stable band for characteristic sulfur emission. With optimized flame conditions, we find a three-fold increase in sensitivity and control of quenching reactions, and this leads to straightforward analysis of different sulfur-containing fuels.

INTRODUCTION

The flame photometric detector (FPD) was first described by Drager and Drager[1] and Brody and Chaney [2,3] more than 20 years ago. There have been relatively few improvements in the detector design over that time period with the exception of the dual-flame detector, described by Patterson[4,5].

The FPD has a complex mechanism which is still not well understood, as evidenced by the recent review of Farwell and Barinaga[6]. They conclude that it is remarkable that the FPD may be used for quantitative work, in view of the complexity of formation, excitation, and quenching of the chemiluminescent species (S_2*) in the flame.

The FPD has been shown to have a number of inherent problems, such as non-linear (exponential) response, variation in the response factor with molecular structure, and quenching by hydrocarbons and other species which are eluted together with the sulfur compounds. Zehner and Simonaitas[7], Aue and Flinn[8], and Cardwell and Marriott[9] have used dopants such as SO_2 or CS_2, to linearize the output of the detector. The difficulty with this procedure is that the dynamic range is reduced and the linear output is dependent on the dopant and the solute. Bradley and Schiller[10] have converted all sulfur compounds to SO_2 in a pyrolysis furnace prior to analysis by the detector in order to eliminate the variability of the exponential re-

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sponse. Sevcik and Phuong Thao[11] have evaluated the selectivity of the FPD and suggested that the unsuitable geometry of the interference filters resulted in interference from hydrocarbons and heteroatoms. Interference filters are effective only when transmitting "well-collimated" light. Most commercial FPDs do not collimate the radiation from the flame. Sevcik and Phuong Thao[11] found that the selectivity of the detector improved with respect to hydrocarbon and heteroatom interference when the flame output was collimated.

In spite of the many problems, the FPD has endured, and the sulfur selectivity is still unmatched by any other detector. We describe a new detector which employs a rare-earth glass filter[12] in place of the interference filter to eliminate some of the problems discussed above.

EXPERIMENTAL

The initial work was conducted with a FPD which was modified by the substitution of a rare-earth glass filter (described in the following section) for the standard interference filter. In addition, the detector inlet was redesigned to minimize the dead-volume. The photomultipler current was monitored with a HNU PI 52 electrometer (HNU Systems, Newton, MA. U.S.A.). The principal gas feeds to the FPD were individually regulated and fitted with flow controllers. Flow meters were calibrated versus orifice plate meters and/or bubble meters.

A HNU Systems gas chromatograph (Model 421) with an integral photoionization detector (PID) was employed in the isothermal mode with either an 8 ft. × 1/8 in. PTFE column packed with acid-washed Haye Sep D or a 10 m × 0.32 mm fused-silica Poraplot Q column (Chrompack, Tahway, NJ, U.S.A.). Samples were injected with a HNU six-port gas sampling valve operated at ambient temperature. The carrier gas was high-purity nitrogen or argon (Liquid Carbonic, Cambridge, MA, U.S.A.). The low-level standard contained four sulfur compounds in nitrogen (Scott, Plumsteadville, PA, U.S.A.). At the temperatures used for the separations, dimethyl disulfide was not eluted during the analysis. All other chemicals used were of ACS grade or equivalent. The chromatographic signals were recorded on a Spectra-Physics integrator (San Jose, CA, U.S.A.) or a Linseis recorder (Princeton, NJ, U.S.A.). The experimental variables, flow-rates, temperatures, etc., are described with the results discussed.

The rare-earth glass filter used was assembled by HNU. The rare-earth glass was designed by E. Snitzer and supplied by BED Corp. (Waltham, MA, U.S.A.). HNU has arranged to fabricate the filter for FPDs of its manufacture.

RESULTS AND DISCUSSION

A new type of optical filter was described by Snitzer[12] which employs rare earth glasses to form a class of absorption type band pass filters. One of the problems with interference filters was discussed by Sevcik and Phuong[11]. They suggested that radiation from the flame, incident on the surface of the interference filter at angles other than 90° can result in a bandpass which is broader than the nominal value. This results in interference from hydrocarbon and heteroatoms. They were able to improve the design through the use of a lens system and a light pipe. Their results for pesticides

(S/P ratios) were in excellent agreement with the theoretical values only when interference filters were properly utilized.

Rare-earth glass filters which function by ionic absorption throughout the bulk of the filter material are not wavelength-dependent on the angle of incidence. Snitzer[12] has shown that the rare-earth groups which have partially filled 4f electron shells can be used to form a large class of bandpass optical filters. A summary of the rare-earth glass absorption regions is shown in Fig. 1. A filter for sulfur was tailored by us to match certain emission lines of S_2*. The filter was composed of several rare earths to define the bandpass. The transmission spectrum is shown in Fig. 2A and can be compared with the transmission spectrum of the conventional interference filter in Fig. 2B. Note that the two primary peaks (Fig. 2A) match sulfur emission lines which are indicated at the top of Fig. 2A. Some of the weaker transmission bands at shorter wavelengths also match the sulfur emission lines. Since both bands in the filter are near the emission maximum for S_2*, one might expect that the sensitivity of the rare-earth filter on the FPD would increase by a factor of *ca.* 2 two under ideal conditions since this filter will allow more S_2 energy to pass through. The results were obtained by substituting filters and keeping the detector operating conditions the same to minimize any operational differences.

The actual signal-to-noise data (RE/IF) found for the two detectors was 1.95 ± 0.1 for the Scott Standard and 2.7 ± 0.1 for the sulfur in propane fuel. The Scott standard concentrations were nominally 4 ppm. The sulfur compound in propane fuel was not otherwise analyzed but is expected to be at about the 5 ppm level (normal for industry practice). There was no apparent difference in the noise level for the two

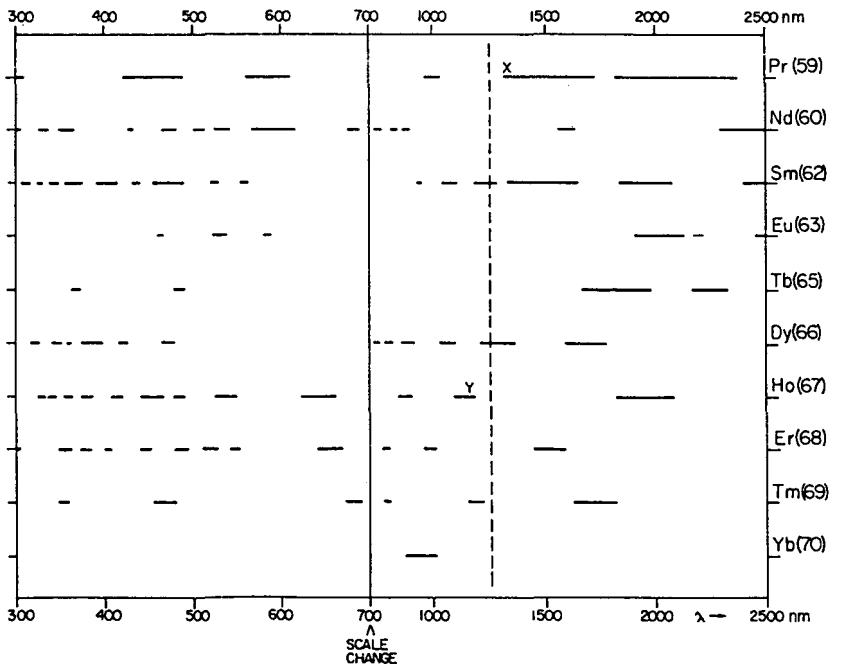

Fig. 1. Summary of rare-earth glass absorption regions (by permission of BED Corp.).

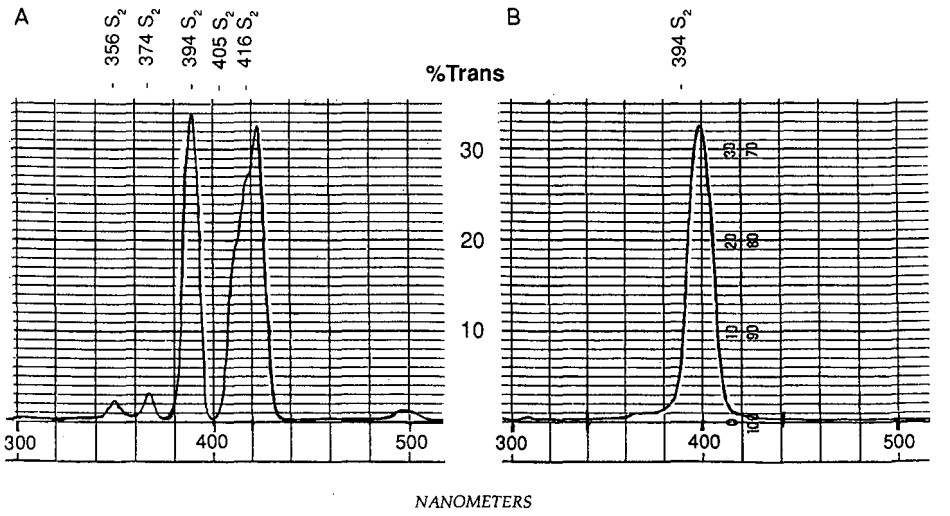

Fig. 2. Comparison of transmission spectra. (A) Rare-earth glass filter; (B) interference filter.

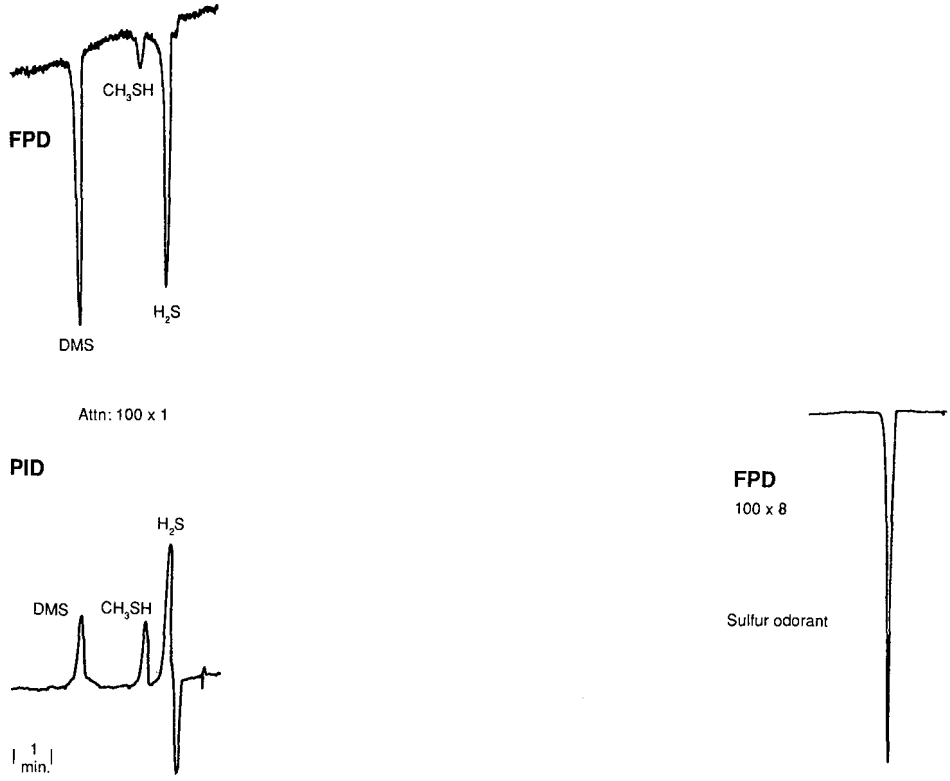

Fig. 3. Chromatogram of sulfur compounds on a Poraplot column. Conditions: 10 m × 0.32 mm I.D. Poraplot Q; detectors in-series; carrier: He, 3 ml/min; oven: 125°C; detectors: 200°C sample loop: 405 μl. Rare-earth glass filter.

Fig. 4. Chromatogram of sulfur in propane fuel with FPD (rare-earth filter) and Poraplot column. Conditions: same as Fig. 3.

detectors operated under the same conditions, so that we were able to achieve the desired improvement in signal-to-noise level that was expected.

We have compared the FPD with the PID for a "clean" sample, a calibration gas, which contains a series of sulfur compounds in nitrogen. The PID was coupled in-series with the FPD downstream. A typical chromatogram for these two detectors is shown in Fig. 3. The amplifier sensitivity for the two detectors was similar, and the chromatograms were similar except for the negative peak in the PID. This peak may be due to a contaminant in the cylinder such as Freon, to which the PID responds but which has no response on the FPD. When the propane fuel gas was used as a sample,

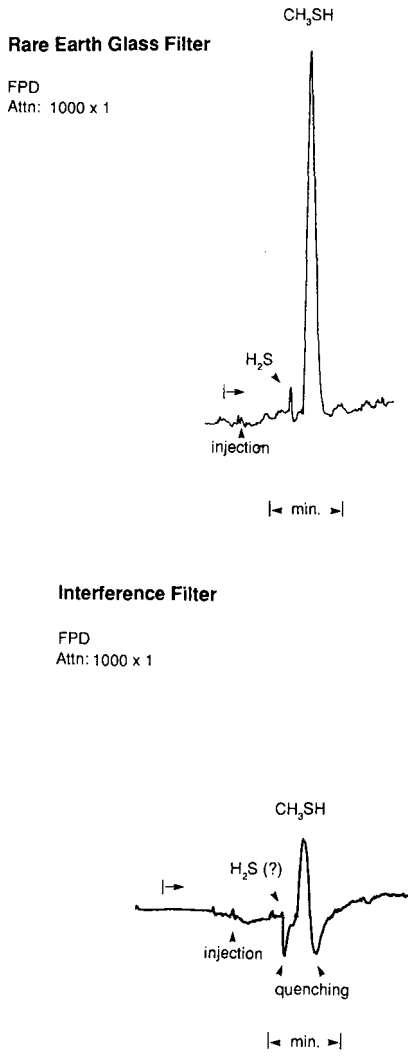

Fig. 5. Comparison of propane fuel with various FPDs (interference filter and rare-earth glass filter). Conditions: 8 ft. × 1/8 in. PTFE, 2 mm I.D., acid washed Haye Sep D packing; carrier: Ar, 29 ml/min; oven: 150°C; detectors: 180°C; sample loop 405 μl.

the optimized FPD with a rare-earth glass filter showed only one peak (Fig. 4) at about the same level as the calibration gas; the PID showed a large off scale response presumably for propylene, an impurity plus a second peak for the odorant (sulfur compound). The problem of sulfur selectivity or lack thereof arises for the PID. In Fig. 5, the results for the same sample (packed column) and the rare earth filter and interference filter FPDs are compared. Note the severe quenching of the response by the hydrocarbon for the interference filter, while the rare-earth filter presents no such problem. In fact, a low-level (ppb) H_2S peak was also observed with this sample.

The rare-earth glasses have a number of additional advantages including the lack of temperature and aging effects common to interference filters. Rare-earth glasses are, in fact, used as wavelength calibration standards by virtue of their inherent stability. Other potential uses of the rare-earth band-pass filters include the measurement of phosphorus, tin, and selenium compounds. Similar improvements in results would be expected with these filters.

CONCLUSIONS

Our optimized rare-earth-filter based FPD appears to have the potential to solve a number of problems from which the detector has suffered since its inception. The use of these filters improves the selectivity by eliminating the problem of angular dependence, which occurs with interference filters. At the same time, the sensitivity of the detector is improved by a factor of 2–3 by observing a number of the S_2 emission lines simultaneously.

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CHROMSYMP. 1566

CHARACTERIZATION OF COAL LIQUEFACTION PRODUCTS BY GAS CHROMATOGRAPHY–FOURIER TRANSFORM INFRARED SPECTROSCOPY–MASS SPECTROMETRY

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SUMMARY

An integrated gas chromatography–Fourier transform infrared spectroscopy–mass spectrometry system was utilized in the analysis of a neutral and acidic fraction obtained from the aqueous portion of the low-severity coal liquefaction products. Reconstructed selected-ion chromatograms along with reconstructed absorbance chromatograms over elected ranges were useful in discriminating between the types of organic compounds in the complex mixtures. A series of γ-lactones and a series of ketones were characterized, the ring size and unsaturation being determined by the absorbance frequency of the carbonyl stretching band.

INTRODUCTION

The integration of capillary gas chromatography (GC) with both Fourier transform infrared spectroscopy (FT-IR) and mass spectrometry (MS) has resulted in a powerful method for the characterization of complex mixtures. The advantages of these linked GC–FT-IR–MS systems have been discussed by several authors[1–4]. The analysis of components separated on the high-resolution capillary column by both IR spectroscopic and MS techniques greatly increases the confidence in identification of the components of the mixture. MS and vapor-phase library search results can be compared to eliminate incorrect or false-positive library identifications. For compounds the spectra of which are not in the libraries, the identification by logical analysis of the two spectra is a tremendous advantage. Furthermore, by examining and comparing the reconstructed chromatograms of selected ions from the stored MS data and the FT-IR absorbance reconstructions over selected frequency ranges corresponding to absorptions of functional groups, the analyst can rapidly assess the compound classes of various components of the complex mixture.

This report describes the use of an integrated GC–FT-IR–MS system for the identification of the volatile components which result from the conversion of coal into liquid and gaseous fuels. Initially our focus has been on the water-soluble products obtained in low-temperature (low-severity) experiments with low-rank coals. The system, developed at the University of North Dakota Energy Research Center, for

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these analyses consists of a capillary gas chromatograph interfaced serially with the light pipe of an FT-IR spectrometer and an ion-trap detector[5]. The serial interface allows the total column effluent to pass through the FT-IR light pipe, thus maximizing the response from the less sensitive FT-IR instrument. Furthermore, the time scale is always the same for both FT-IR and ion-trap spectrometers, just shifted by a constant number of seconds. The amount of column effluent entering the mass spectrometer is readily adjusted by varying the make-up gas flow to the light pipe. Hydrogen carrier gas is used for better chromatographic performance. The FT-IR spectrometer is equipped with a coprocessor which rapidly transforms the interferograms and allows spectra to be stored on the disk along with part of the interferogram. Absorbance reconstruction of chromatograms can then be accomplished in a reasonable time from the stored spectra.

EXPERIMENTAL

The GC–FT-IR–MS system was assembled with a Nicolet 20SXB FT-IR spectrometer and a Finnigan 700ITD, connected serially via an open-split interface, as described previously[5].

The low-severity coal liquefaction conditions and apparatus utilized for production of the products described in this paper will be described elsewhere. Prior to the GC–FT-IR–MS analysis, the organic products in the aqueous layer resulting from liquefaction of Beulah lignite at 325°C were first extracted into dichloromethane and then separated into a neutral and acidic fraction by extraction of the dichloromethane extract with sodium hydroxide solution. The acid fraction recovered by acidification of the base extract and extraction into dichloromethane was analyzed by on-column injection on the GC–FT-IR–MS system.

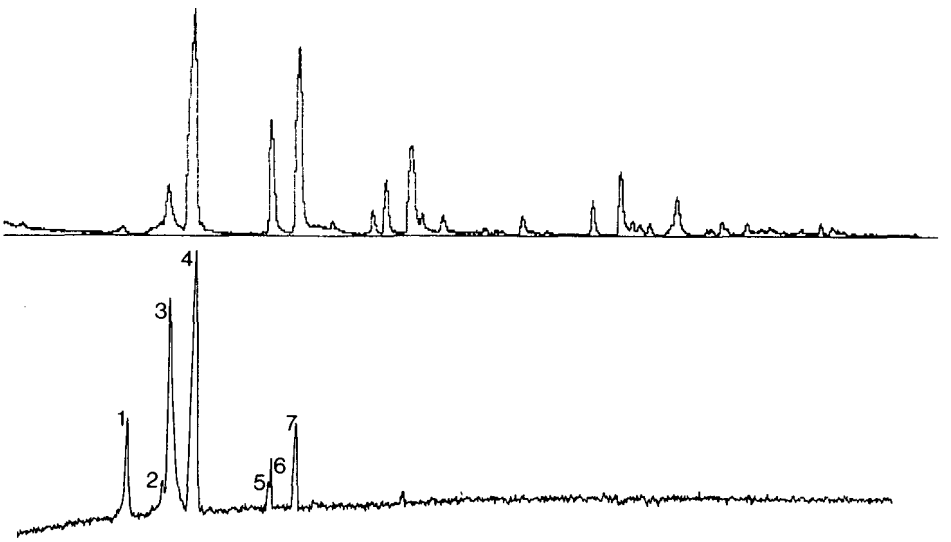

Fig. 1. Chromatograms of the acidic fraction: (top) total ion reconstruction; (bottom) Gram–Schmidt reconstruction. For peaks, see text.

RESULTS AND DISCUSSION

The total ion reconstructed chromatogram and the Gram–Schmidt (FT-IR) reconstructed chromatogram[6] of the acidic fraction from the low-severity products (Fig. 1) showed somewhat different responses to the components in the sample. The more intense peaks in the Gram–Schmidt reconstructed chromatogram were phenol and cresols (peaks 4, 5 and 7), which were present in high concentrations, and a series of lactones (peaks 1, 2, 3 and 6), which have not been previously identified in liquefaction products. Other phenolics present in lower concentrations appeared in the reconstructed ion chromatogram but not in the Gram-Schmidt reconstruction.

Characterization of the lactone series was aided by examining the absorbance-reconstructed chromatogram, which was obtained for the range 1800–1900 cm^{-1} (Fig. 2). The absorbance maxima for all of the lactones were actually between 1808 and 1818 cm^{-1}, indicating that the lactones are γ-lactones. In this fraction, no carbonyl absorptions were found for the range 1700–1800 cm^{-1}, indicating that there were no δ-lactones present, and that the ketones which were present in the neutral fraction (see discussion below) had not been extracted into the basic solution so as to contaminate the acidic fraction.

γ-Butyroactone and γ-valerolactone (peaks 1 and 3 in Fig. 2) were identified by matching FT-IR library spectra. The mass spctra of these components were identical

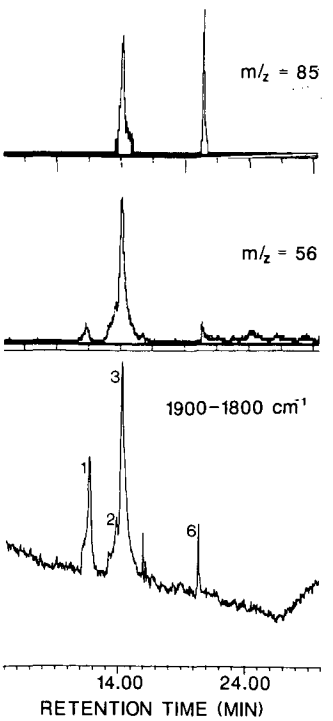

Fig. 2. Chromatograms of the acidic fraction: (top) m/z 85 reconstruction; (middle) m/z 56 reconstruction; (bottom) absorbance-reconstruction over 1800–1900 cm^{-1}. For peaks, see text.

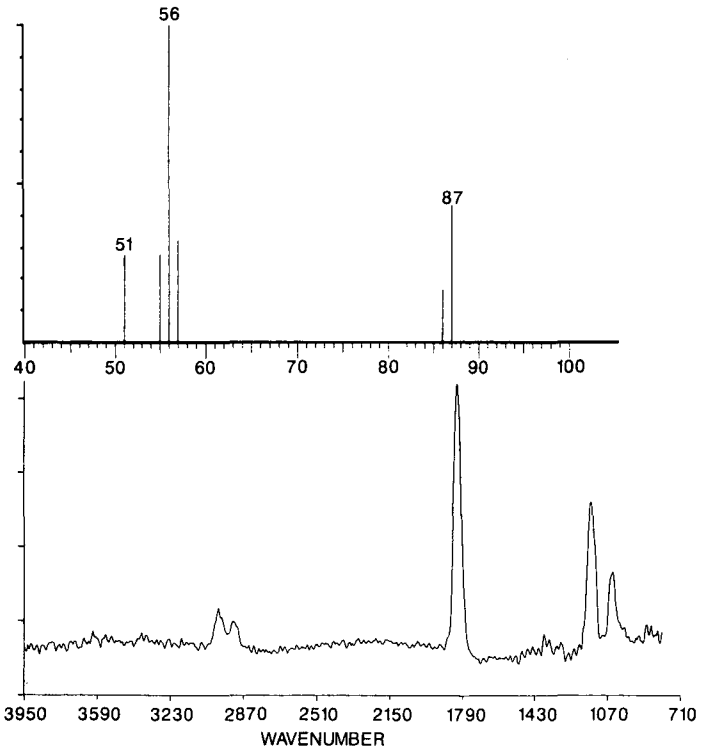

Fig. 3. Spectra for peak 1 in Fig. 2 (γ-butyrolactone): (toi) mass spectrum (ion-trap detector); (bottom) FT-IR spectrum.

to library spectra, except that the ion-trap spectra of the butyrolactone (Fig. 3) and valerolactone exhibited $(M + 1)^+$ ions at m/z 87 and 101, respctively, in addition to the M^+ ions at m/z 86 and 100. The original commercial ion trap (Finnigan 700ITD) used in these studies was subject to self-chemical ionization (self-CI) effects for certain classes of compounds[5]. Because of the relatively high source pressure, certain oxygen-containing and other basic compounds were observed to undergo protonation and other adductive reactions with the large concentration of ions present during the storage cycle. Thus, esters and amines frequently exhibited the $(M + 1)^+$ ions in their spectra. The $(M + 1)^+$ ions were shown be narrow peaks, rather than the broad type of peak which can result from space-charging and saturation in the ion trap and whose masses are then incorrectly assigned by the data system. When the M^+ ion is absent from the spectrum, observation of the $(M + 1)^+$ ions can then give molecular weight information, as in CI-MS with reagent gases.

Fragmentations and self-CI protonation for γ-butyrolactone and γ-substituted lactones are shown in Figs. 4 and 5. The γ-alkyllactones characteristically lose the alkyl group to give the m/z 85 fragment ion. Loss of the γ-carbon with its attached atoms results in the m/z 56 cyclopropanone ion. Selected-ion reconstructions for m/z 85 and m/z 55 will discriminate the lactones on the basis of positional isomerism (or ring size. if

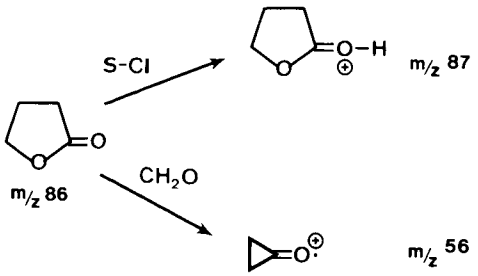

Fig. 4. Fragmentation of γ-butyrolactone.

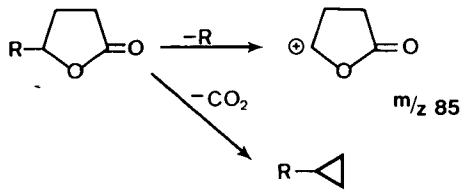

Fig. 5. Fragmentation of γ-alkyllactones.

other lactones were present). The *m/z* 85 and 56 reconstructions for the acidic coal liquefaction sample are shown in Fig. 2, along with the absorbance reconstruction.

Besides the butyrolactone and valerolactone, two other lactones were present in small amounts, peak 2, which lacked the *m/z* 85 ion, and peak 6, which exhibited the *m/z* 85 ion. The retention time and mass spectrum of peak 2 matched those of α-methyl-γ-butyrolactone. However, the β-methyl isomer cannot be ruled out, since the mass spectrum of this lactone would also lack the *m/z* 85 ion. The β-isomer or its library FT-IR spectrum or mass spctrum were not available for comparison. Peak 6, which was partially separated from *o*-cresol (peak 5) was identified as γ-ethyl-γ-butyrolactone because of the $(M + 1)^+$ ion at *m/z* 115, the large fragment ion at *m/z* 85 (base peak) which resulted from loss of γ-ethyl radical, and the lack of *m/z* 99 ion, characteristic of the dimethyl-substituted butyrolactones.

The phenolic components of the sample were easily identified by the mass spectra and the retention times. This aspect of the analysis was routine, and since only the usual phenolic products found in coal-derived liquors were identified, the results will not be discussed here. Except for phenol and cresols, the FT-IR spectra of the phenolics from this set of data were not usable due to a low signal-to-noise ratio. However, a more concentrated sample gave spectra which confirmed the phenol identification. Only trace levels of guaiacols and catechols were present in this sample.

Characterization of the neutral fraction by GC–FT-IR–MS showed that a large variety of polar organic components were present (Fig. 6). These components were identified as cyclic alcohols, cyclic ketones and unsaturated cyclic ketones. A trace of the phenolics and one of the lactones has carried over into this fraction as a result of incomplete extraction. This is a common problem in the analysis of aqueous coal-derived materials, which requires continual inspection. Absorbance-reconstructed chromatograms for the carbonyl region (1700–1800 cm^{-1}) and reconstructed chromatograms of *m/z* 55 fragment ions were highly selective for the cyclic ketones.

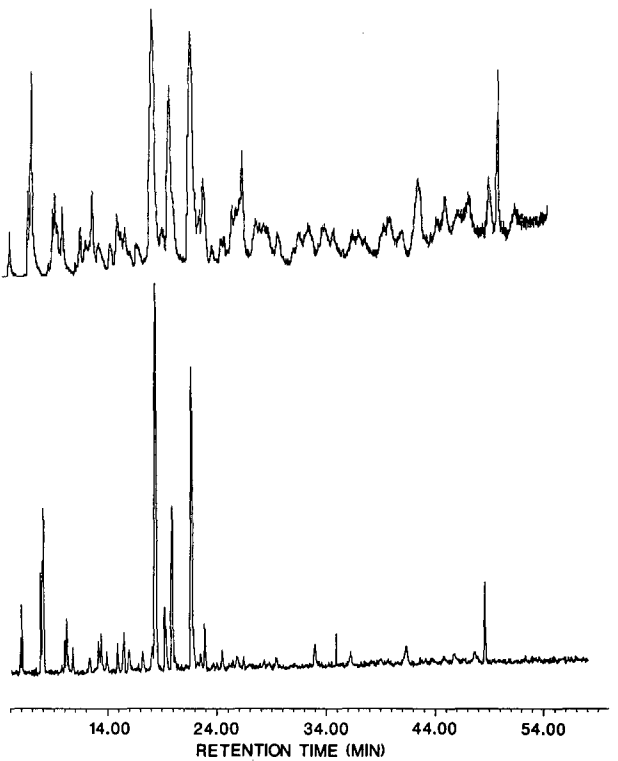

Fig. 6. Chromatograms of the neutral fraction: (top) total-ion reconstruction; (bottom) Gram–Schmidt reconstruction.

The unsaturated cyclic ketones were identified by the reconstructed chromatograms of *m/z* 53 ions and the carbonyl absorbance reconstruction. Reconstructed ion chromatograms of *m/z* 57 iobs and absorbance-reconstructed chromatograms for the hydroxyl stretching region identified the cyclic alcohols.

The first portions (12 min) of these reconstructed chromatograms are shown in Fig. 7. From the 1700–1800 cm^{-1} absorbance reconstruction, peaks 2, 3, 5, 7, 8, 9, 10 and 11 were characterized as cyclic ketones. These characterizations were confirmed for peaks 2, 3, 5, 7, 8, 9 and 11 by examining the *m/z* 55 ion chromatogram and for peak 10 by examining the *m/z* 53 chromatogram. Each component was then identified from the individual IR and mass spectrum. IR spectra showed that peaks 2, 3 and 5 were cyclopentanones (1759 cm^{-1}) and that peak 10 was a cyclopentenone (1737 cm^{-1}) (Fig. 8). Thus peaks 2, 3 and 5 were cyclopentanone (M^+ at *m/z* 84), 2-methylcyclopentanone, and 3-methylcyclopentanone (M^+ at *m/z* 98). Mass and FT-IR spectra of these components matched library spectra. Peaks 7, 8, 9 and 11 were C_2-cyclopentanone isomers (M^+ at *m/z* 112), the structures of which could not be further elucidated because of lack of standards and library spectra. Peak 10, which was partially separated from the C_2-cyclopentanone, was identified as a methylcyclopentenone from the mass spectrum (M^+ at *m/z* 96). The mass spectrum from peak 10 (Fig. 8) did not match the U.S. Environmental Protection Agency/National Institutes

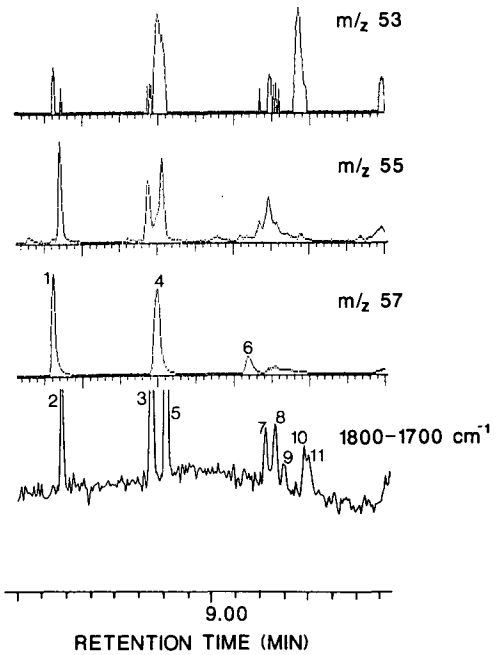

Fig. 7. Chromatograms of the neutral fraction: (top) *m/z* 56 reconstruction; (upper middle) *m/z* 55 reconstruction; (lower middle) *m/z* 57 reconstruction; (bottom) 1700–1800 cm^{-1} absorbance reconstruction. For peaks, see text.

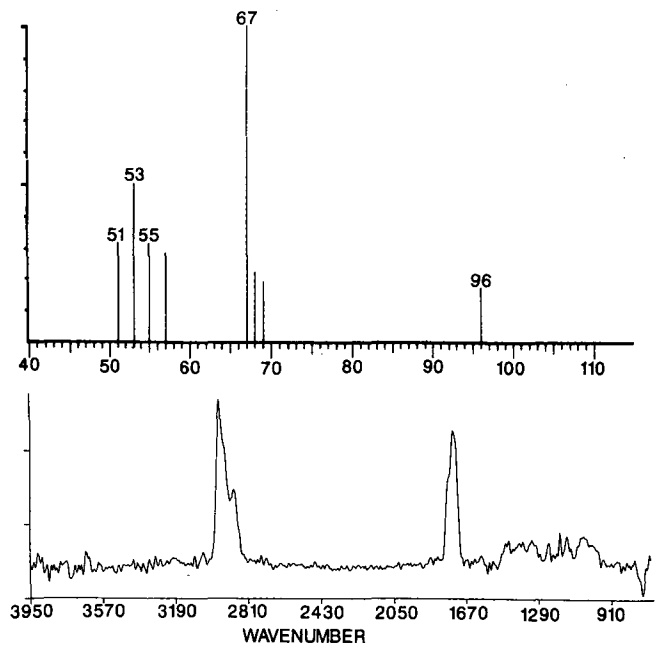

Fig. 8. Spectra for peak 10 in Fig. 7 (methylcyclopentanone): (top) mass spectrum; (bottom) FT-IR spectrum.

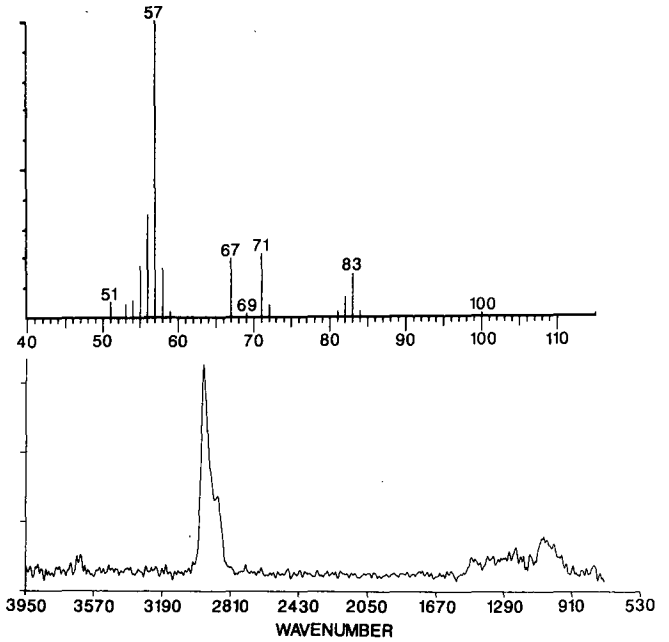

Fig. 9. Spectra for peak 4 in Fig. 7 (2-methylcyclopentanol): (top) mass spectrum; (bottom) FT-IR spectrum.

of Health library spectra of 2-methyl- or 3-methylcyclopentenone. More highly substituted alkyl-cyclopentenones and indanones were eluted later in the chromatography of this sample.

The alcohols in the sample were distinguished by the *m/z* 57 reconstructed-ion chromatogram (peaks 1, 4 and 6 in Fig. 7). The IR spectra of these peaks exhibit the hydroxyl-stretching absorption at 3650 cm^{-1}, which is characteristic of alcohols, and none have the carbonyl absorption, which clearly establishes that none are lactones or ketones. The IR spectrum of Peak 4 is shown in Fig. 9 as an example. The three alcohol components (peaks 1, 4 and 6) were identified as cyclopentanol, 2-methylcyclopentanol, and cyclohexanol by matching library FT:IR and mass spectra. The mass spctra of the three alcohols show very weak or no molecular ions and small $(M - 17)^+$ or $(M - 18)^+$ ions. For example, the mass spectrum of peak 6 had an ion at *m/z* 82 but no molecular ion at *m/z* 100. The characterization of these alcohols was aided considerably by having both IR and MS data on each component peak. No alcohols larger than cyclohexanol were present in the sample.

Many of the components of the liquefaction samples either were compounds whose spectra were not in the library or were isomers having similar spectra. Examination of both the MS and FT-IR spectra aided in the identification. Improvements in the analysis will result from greater FT-IR sensitivity, which will improve the spectra of the smaller peaks and allow us to utilize more of the weak but highly diagnostic IR bands in the identification.

ACKNOWLEDGEMENTS

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CHROMSYMP. 1547

ELEMENT-SELECTIVE GAS CHROMATOGRAPHIC DETECTION BY ATOMIC PLASMA EMISSION SPECTROSCOPY

REVIEW AND DEVELOPMENTS

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SUMMARY

The recent progress and current developments in element-specific detection for gas chromatography by atomic plasma emission spectroscopy are reviewed with emphasis on the applications of microwave-induced plasmas (MIPs) in capillary separations. A number of current applications of atmospheric-pressure MIP detection are then considered, including empirical formula measurement of cyclosiloxane pyrolyzates from silicones, ligand-exchange reactions of aluminum chelates, selectivity and sensitivity considerations in rhodium-specific detection, and dual-element detection for boron and iron in ferroceneboronate derivatives of diols.

INTRODUCTION

Chromatographic detectors may be "universal", "selective", or "specific". Detection of all eluted peaks is desirable for some applications, but it is often necessary to discriminate specific components in an incompletely resolved chromatogram or to carry out the analysis of one particular analyte class. Detectors may be "element-selective", "structure- or functionality-selective", or "property-selective". Specific detectors are those exhibiting a sufficiently high amount of selectivity to be effectively "blind" to eluates other than the target analyte. The separating capability of the chromatographic column can thus be complemented by selective detection to achieve enhanced analytical resolution.

The objectives of element-selective chromatographic detection are qualitative and quantitative measurement and identification of eluates, based upon elemental content; simultaneous multi-element detection may enable empirical formulae of eluates to be determined. Some element-selective detection methods are in common use in gas chromatography (GC): alkali flame ionization detection (AFID) often known as nitrogen–phosphorus detection (NPD), flame photometric detection (FPD), which is selective for sulfur and phosphorus, and Hall electrolytic conductivity detection (HECD), which is selective for halogens, nitrogen, or sulfur. In high-performance liquid chromatography (HPLC), element-selective detectors have been

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little used. Atomic emission spectroscopy is a powerful method for such interfaced detection because of its capacity to monitor all elements; much recent effort has focused on its employment for on-line chromatographic detection. Various modes of atomic spectroscopy have been interfaced for GC detection, atomic absorption (AAS), flame emission (FES) atomic fluorescence (AFS) and atomic plasma emission. All these methods have also been used in HPLC detection. The scope of that work is summarized in a recent review[1].

THEORY

Survey of plasma emission GC detection

Inert gas plasma emission methods have strong analytical capability. Although sophisticated spectroscopic instrumentation is needed for simultaneous multi-element detection, sequential single-element detection can be achieved quite simply. Various atom reservoirs were developed to produce emission spectra from atoms rather than molecules, thereby enhancing detection selectivity. Complete molecular fragmentation also allows element rationing and empirical formula measurement[2]. The major plasma sources used in GC detection have been the microwave-induced helium plasma, at atmospheric or reduced pressure, the direct current argon plasma (DCP) and to a lesser extent the inductively coupled argon plasma (ICP). The microwave-induced plasma (MIP) system is the most economical to operate with respect to plasma gas usage and power requirements. The advantages of plasma emission detection include the ability to "speciate" for many metals and non-metals, either directly or by derivatization through suitable chemical reactions. (The term speciate involves determination of the specific molecular species in which the analyte elements occur.) There is also the ability to tolerate non-ideal chromatographic elution, to achieve high elemental sensitivity and carry out multi-element monitoring.

The major atom reservoirs used for GC detection have been microwave-indiced electrical discharges (plasmas). An argon or helium plasma is maintained within a microwave cavity, which focuses coupled power from a microwave source, usually operated at 2.45 GHz, into a sample discharge cell. The spectral emission is focused onto the entrance slit of a spectrometer; the detection system may consist of a photomultiplier tube, a number of tubes in the case of polychromators, or increasingly, diode arrays. The most frequently used plasma cavity for GC has been an atmospheric pressure device, sustained in a TM_{010} cavity of the type first described by Beenakker[3]. Other frequently used plasma sources have been reduced pressure cavities of the type introduced by Fehsenfeld *et al.*[4], which can also be used at atmospheric pressure for an argon plasma gas. The efficiency of the MIP also depends on waveguides, metal tubes which transfer power from a microwave generator to the plasma gas. If the waveguide configuration is "interrupted" to give total reflection of energy traveling along it, "standing waves" are set up to produce a "resonant cavity". A comparison of cavities for GC detection was made by Risby and Talmi[5]. Bache and Lisk[6] were among the first to employ a plasma detector to determine ng levels of Br, Cl, I, P and S in organic eluates. The reduced-pressure GC–MIP system was shown effective for P, S, Br, Cl, I, C, H, D, N, O and other elements by McLean *et al.*[7].

Line emission is observed for almost all elements in the helium plasma, giving an advantage for selective detection over the less-energetic argon MIP, in which such

elements as Cl, Br, N and O give weak diatomic molecular emission. GC detection limits were found in the 0.03–0.09 ng/s range[7], but the best selectivities against carbon were around 2000:1. This instrumental system was the precursor of commercial GC–MIP instrumentation[8] for which Brenner[9] evaluated the sensitivity, selectivity and reproducibility. Kewei *et al.*[10] have used a similar polychromator instrument for oxygen-specific detection; purified plasma gases and air exclusion from the system helped to optimize the detection limit at 0.3 ng/s.

Hagen *et al.*[11] used chlorodifluoroacetic anhydride derivatization to introduce element taggants F and Cl into amines to obtain improved GC–MIP selectivity and sensitivity. Olsen *et al.*[12] compared reduced-pressure and atmospheric-pressure MIP for detection of organomercury, selenium and arsenic. They found the latter system better, giving 1-pg detection for mercury with selectivity over carbon of 10 000. Quimby and Sullivan[13] described a cooled modified Beenakker cavity with exit-gas purge, a moveable diode array and a holographic concave grating for sensitive multi-element monitoring.

The applications of MIP in GC detection have been extended recently in various ways. Pivonka *et al.*[14] used a 370-W water-cooled Beenakker MIP system and a tangetial-flow torch[15] to gather time-resolved interferograms with a Fourier Transform near-infrared (FT-NIR) emission spectrometer. Computer-generated element specific chromatogram reconstructions for C, H, N, O, F, Cl, Br and S were obtained. The "surfatron" MIP cavity has been usefully applied for GC detection. Takigawa *et al.*[16] constructed a versatile multi-element detector by combining it with a photodiode-array spectrometer to give an isometric spectrochromatographic display of data. Rivière *et al.*[17] used a surfatron monochromator system to obtain low pg/s detection for Cl, P and S in pesticides. The surfatron has also proven viable as a detector for supercritical-fluid chromatography (SFC), affording sulfur-specific detection at the 25-pg/s limit for thiophene[18]. Extensive spectral characterization was carried out for this system for two common SFC mobile phases, carbon dioxide and nitrous oxide[19]. Helium afterglow[20] and radiofrequency plasma discharge detectors[21] have also been found useful for capillary GC detection.

For wide application of plasma emission chromatographic detection suitable instrumentation must be readily available. Thus, the atmospheric-pressure DC argon plasma (ARL Spectraspan), together with a high-resolution echelle monochromator, has been used for GC detection (GC–DCP)[22] for metals and for elements in GC-derivatizing groups such as boron and silicon. Capillary GC detection limits obtained were: for Cr 4 pg/s and selectivity over carbon $4 \cdot 10^8$, for Sn 60 pg/s and $2.5 \cdot 10^6$, for Pb 100 pg/s and $5 \cdot 10^5$ and for B 3 pg/s and $3 \cdot 10^5$ (ref. 23). For individual analyses, GC–DCP and GC–MIP systems have contrasting advantages. The latter is well suited for capillary GC, and its excellent detection limits allow trace determinations of very small samples. However, it has a limited capacity for larger samples, and packed-column applications require solvent venting. The DCP system is somewhat less convenient but accomodates a wider range of sample sizes. For many metals and metalloid elements, the sensitivities of the DCP are close to those of the MIP, and selectivities over carbon are very high.

Despite its wide use as a spectroanalytical emission source, the ICP has seen little use as a GC detector. Windsor and Denton[24] focused their attention upon the elements Br, Cl, F, I, H, Si and C and the metals Sn, Pb and Fe. Detection limits for the latter

were around the ng level, as were those for C and H, but those for F, Cl and Br were at or above the μg level.

Multi-element detection and empirical formula determination

Among the most attractive attribution of element-specific detection is the potential to determine element ratios and, thence, empirical formulae of resolved components at low sample levels. Results and conclusions of various investigators have differed. While some have questioned such determinations because of apparent element response dependency upon molecular structures, and poor detection limits and linearity[25], others have found precision and accuracy to within 1–2% for compounds of similar chemical composition. Thus, Perpall *et al.*[26] used a multi-referencing technique from pyrolysis of known polymers to provide a reproducible range of reference compounds. Linear alkanes, alkenes and alkadienes, produced by pyrolysis of high-density polyethylene, gave relative errors between the actual formula and the experimental formula of 6.7% or less, over the range of C_8 to C_{20} regardless of the hydrocarbon reference chosen. *i.e.* the unknown and the reference compounds did not need to be close in molecular weight. By contrast, these authors found that for pyrolysis of polydimethylsiloxane (PDMS) to a homologous series of cyclic dimethyl-siloxanes, only a narrow reference window could be used, corresponding to one or two homologues, if accurate empirical formulae were to be obtained. Thus there was a need to determine whether the elemental responses for these siloxanes were generally dependent on molecular size and weight of the compound. To accomplish this goal, pyrolysis of an amine-substituted siloxane was carried out, as described below, and empirical formulae of siloxane pyrolyzates of different sizes were calculated. This investigation and other recent studies in our laboraroty, involving operational considerations for different elemental measurements, serve to illustrate the usefulness of this developing technique.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard (Avondale, PA, U.S.A.) 5830A gas chromatograph was used for the pyrolysis experiments with a Hewlett-Packard HP 18835 capillary inlet for split injection. The other injection port supplied helium make-up gas to the interface to induce and sustain the plasma. The capillary column used was 22 m × 0.31 mm I.D., 0.52-μm film thickness, cross-linked 5% phenylmethyl silicone gum (Hewlett-Packard). The atmospheric-pressure multi-channel GC–MIP spectrometer system was as previously desxribed, incorporating a Beenakker type cavity[27], with a modified column–cavity interface to facilitate selctive gas purging[28]. An alumina discharge tube, secured inside a larger quartz tube, was employed for quantitative detection of silicon in the organosilicon compounds. The atomic emission lines selected for this study were: carbon, 247.86 nm, 2nd order; hydrogen, 656.28 nm, 1st order and silicon, 288.16 nm, 2nd order. The pyrolysis was carried out in a Chemical Data Systems (Oxford, PA, U.S.A.) Model 120 Pyroprobe coil-type pyrolyzer with a quartz tube insert. The pyrolysis interface temperature was set to 245–250°C to minimize mass discrimination or thermal decomposition therein. Element-specific responses were displayed on two dual-channel D-4000 Omniscribe chart recorders (Houston Instru-

ments, Austin, TX, U.S.A.), and peak areas were measured by a Model 18850A terminal (Hewlett-Packard).

For inorganic GC, a single-channel GC–MIP system, incorporating a Hewlett-Packard 5840A gas chromatograph and a direct capillary column interface to a Beenakker cavity, was used[30]. A high-resolution grating monochromator[31] or a low-resolution scanning monochromator, having *ca.* 0.1 nm resolution[32] were used, as described previously.

Materials

The amine-substituted PDMS, X2-8124, was obtained from Dow Corning (Midland, MI, U.S.A.). The amine mol percentage was given as 8% by the manufacturer, and the number of silicon-based monomer units in the polymer unit averaged 100.

The chelates, aluminum trifluoroacetylacetonate [$Al(TFA)_3$] and aluminum trifluoroacetylpivalonylmethanate [$Al(TPM)_3$] were prepared by reaction of aluminum nitrate with the respective ligands trifluoroacetylacetone (HTFA) and trifluoroacetylpivalonylmethane (HTPM) (Columbia Organic Chemicals, Columbia, SC, U.S.A.), followed by vacuum sublimation of the products. Cyclopentadienyl rhodium dicarbonyl was obtained from M. D. Rausch of the University of Massachusetts (Amherst, MA, U.S.A.). The samples of derivatized ferrocene boranoates were obtained from C. J. W. Brooks of the University of Glasgow (Glasgow, U.K.).

RESULTS AND DISCUSSION

Empirical formula determination

Simultaneous silicon, hydrogen and carbon element-specific MIP pyrograms were obtained upon pyrolysis of a 500-μg sample of the amine-substituted silicone. Fig. 1 shows pyrograms obtained by heating the sample to 650°C at the maximum rate available for the coil probe (*ca.* 1000°C/s), and holding for 10 s. The column temperature was programmed at 6°C/min from 60 to 300°C. Peaks 1 and 2 are hydrocarbons, peaks 3–8 are the cyclic dimethylsiloxanes, D3–D8, and peaks 9–11 are cyclic siloxanes, incorporating an amine substituent. In earlier work[26] the following expression for the calculation of the element (E)-to-carbon (C) ratio had been used

$$\frac{E}{C} = \frac{\text{E atoms in reference}}{\text{C atoms in reference}} \cdot \frac{\text{E (response of unknown)}}{\text{E (response of reference)}} \cdot \frac{\text{C (response of reference)}}{\text{C (response of unknown)}}$$

This assumed that the sensitivity response factors for each element were equal to unity for all reference compounds. This appeared to be valid for the polyethylene pyrolyzates but not for the silicone pyrolyzates in that study, as was discussed earlier. In the present study, the above equation was modified in two ways. Responses were measured by peak areas rather than peak heights, and each response ratio was modified by including as an exponent the appropriate sensitivity constant for the reference compound, D3, used in the calculation. The sensitivity constants employed were 0.7113 for carbon, 0.7626 for silicon and 0.6982 for hydrogen, obtained from calibration curves measured independently for each element for D3[28]. Table I presents

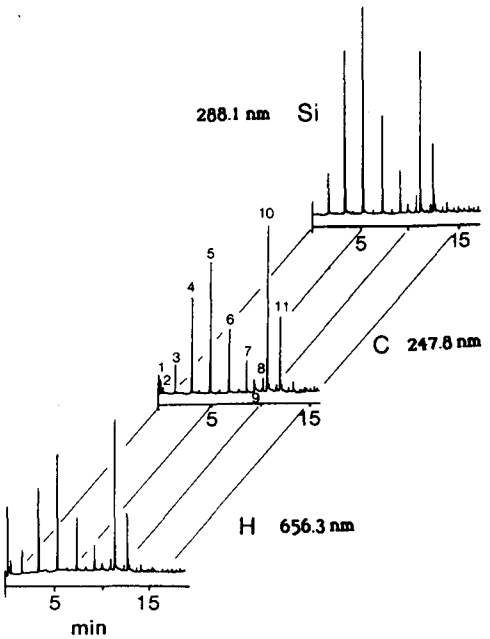

Fig. 1. Simultaneous silicon-, carbon-, and hydrogen-specific detection of pyrolyzates from 0.5 mg of X2-8124 amine-substituted PDMS. Pyrolysis temperature 650°C, held for 10 s. GC column and conditions as in the text, programmed from 60 to 300°C at 6°C/min. Injection temperature, 200°C; pyrolysis interface temperature 300°C. MIP operating conditions: 48 W, helium flow-rate 80 ml/min.

the empirical formulae calculated by this method. It is apparent that the carbon and hydrogen values, referred to silicon, decrease with increasing molecular weights resulting from an increased number of repetition units.

It has been suggested previously that some high-molecular-weight compounds may be incompletely fragmented in the MIP[25]. Our earlier study[26] indicated such a phenomenon for the silicone pyrolysis, and the present results substantiate this

TABLE I

EMPIRICAL FORMULA DETERMINATION OF DIMETHYLCYCLOSILOXANES, PRODUCED BY PYROLYSIS OF X2-8124 AMINE-SUBSTITUTED POLYDIMETHYLSILOXANE

Compound	*Empirical formula*[a] $Si_1C_{2p}H_{6q}O_1$	
	p	*q*
D_3[b]	1.00	1.00
D_4	0.97	0.96
D_5	0.94	0.94
D_6	0.92	0.91
D_7	0.91	0.90
D_8	0.90	0.89

[a] Reference peak.
[b] Theoretical empirical formula is $Si_1C_2H_6O$.

conclusion. It would be of interest to assess the ability of a post-column pre-plasma pyrolysis unit, as designed by Chiba and Haraguchi[29], to overcome this problem.

GC–MIP detection in inorganic reaction chemistry

A recent study of ligand interchange between aluminum, gallium and indium complexes has emphasized the value of element specific GC detection for following such reactions[30]. As a further example of element-specific GC detection, Figs. 2 and 3 show carbon and aluminum specific chromatograms of a solution of two aluminum chelates, $Al(TFA)_3$ and $Al(TPM)_3$, before and after ligand redistribution in dichloromethane solution at 25°C for 24 h. It is of interest to note that the peak shapes observed for the aluminum chelates at the aluminum line at 396.2 nm, exhibit less tailing than those measured at the carbon line. This suggests that peak broadening in the carbon channel reflects memory effects in the plasma rather than chromatographic interactions on the column.

GC–MIP has proved valuable in the study of volatile organometallic compounds. Now reported are initial studies on rhodium-specific detection, which has not previously been described. The emphasis here is on the choice of the emission line, which may be dictated by either sensitivity or selectivity considerations. Rhodium lines, suited for GC monitoring, were established first by bleeding a low level of volatile cyclopentadienyl rhodium dicarbonyl $[CpRh(CO)_2]$ into the MIP at a constant rate. The two preferred spectral lines for analysis proved to be at 343.5 nm [Rh(I)] and 249.1 nm [Rh(II)]. The GC–MIP parameters were optimized by repetitive injections into the

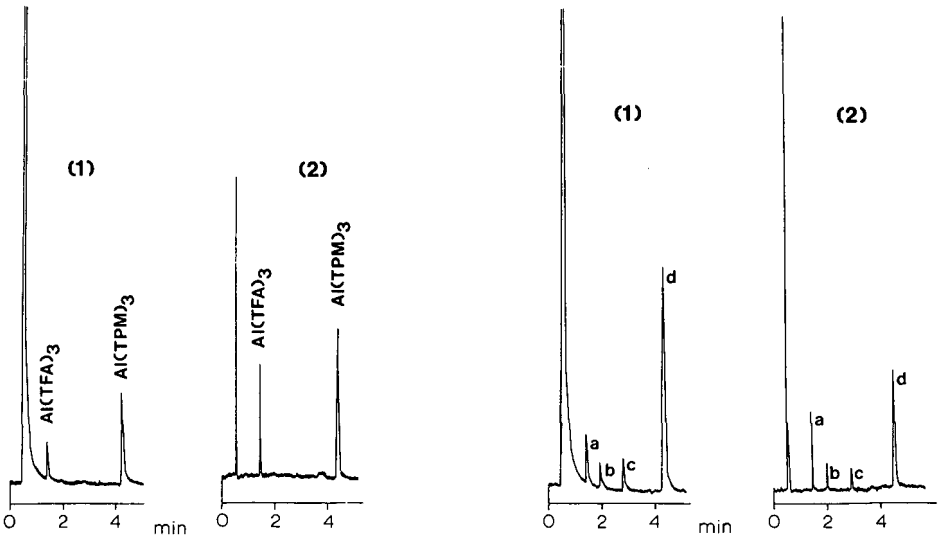

Fig. 2. Dual sequential carbon [(1), 247.8 nm] and aluminum [(2), 396.2 nm] detection of aluminum trifluoracetylacetonate and trifluoroacetylpivalonylmethanate. Column, 10 m × 0.25 mm I.D. SE-30 (fused-silica open-tubular); column temperature, 130°C.

Fig. 3. Dual sequential carbon [(1), 247.8 nm] and aluminum [(2), 396.2 nm] detection of redistribution products of aluminum chelates after 24 h in dichloromethane at 25°C. Column and conditions as for Fig. 2. Key to peaks: a = $Al(TFA)_3$; b = $Al(TFA)_2(TPM)$; c = $Al(TFA)(TPM)_2$; d = $Al(TPM)_3$.

capillary column. The linear response range at 343.5 nm was more than three orders of magnitude, and the detection limit at a signal-to-noise ratio of 3 was 211 pg of the metal.

In Fig. 4 is shown a comparison of chromatograms for the two most useful wavelengths for rhodium emission. The test solution contained 0.34 g of *n*-nonane and 0.067 g of the rhodium compound dissolved in 1 ml of hexane. Selectivity over carbon at 343.5 nm was *ca.* 1100, based on an *n*-nonane response in the linear range. However, the selectivity at 249.1 nm was in the region of 4000, whereas the sensitivity was approximately one order of magnitude less that for the longer wavelength. The choice of emission wavelength here depends on the relative importance of detection limits and the nature of the sample matrix.

Element-specific functionality derivatization

The idea of chromatographic analysis for chemical functionalities through their derivatization with a reagent, containing a particular analyte element or elements, has been introduced earlier[11]. We have undertaken a preliminary exploration of such an approach for cyclic ferroceneboronate derivatives. Brooks and Cole[33] have shown these to be produced quantitatively from reactions of ferroceneboronic acid with 1,2- and 1,3-diols, these compounds giving good GC peak characteristics. A GC–MIP investigation of the same pinacol and dicyclohexyl-1,1′-diol ferrocene boronates as were studied with GC–MS by Brooks and Cole[33] showed a typical pair of chromatograms as in Fig. 5. A mixture of the derivatives at a molar ratio of 0.730 gave responses for iron at 259.94 nm and boron at 249.68 nm. Hydrogen was doped into the

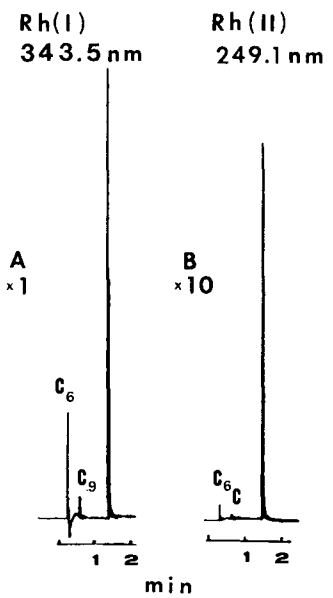

Fig. 4. GC–MIP detection of rhodium in rhodium cyclopentadienyl–dicarbonyl at 343.5 and 249.1 nm. Column, 11 m × 0.20 mm I.D. SE-30 (fused-silica open-tubular); column temperature, 110°C. MIP operating conditions: 70 W, helium flow-rate 500 ml/min. The sensitivity setting for B is ten times that of A.

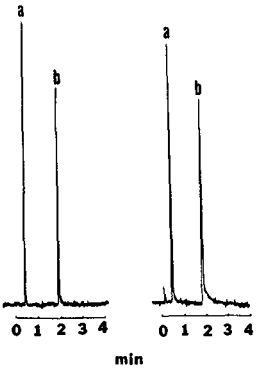

Fig. 5. Dual sequential iron (left, 259.94 nm) and boron (right, 249.68 nm) detection of pinacol ferroceneboronate (a) and dicyclohexyl-1,1'-diol ferroceneboronate. Column, 12 m × 0.32 mm I.D. DB-5 (fused-silica open-tubular); column temperature, 210°C for 30 s, then programmed at 30°C/min to 260°C.

plasma at *ca.* 1 ml/min to reduce peak tailing for the boron response, attributable to deposition of boron in the plasma-discharge tube[34]. Table II shows peak area ratios for iron and boron responses at different sample levels, indicating a consistent elemental response. In this case, boron and iron appear to show very similar absolute sensitivities, although the iron line may be preferred for quantitative studies because of the better GC peak shapes.

The potential utility of this type of elemental derivatization is considerable, particularly when the elements introduced into the analyte molecules, here the diols, are unlikely to be encountered in the remainder of the chromatogram.

TABLE II

RESPONSE RATIOS (PEAK AREAS) FOR PINACOL FERROCENEBORONATE AND DICYCLOHEXYL-1,1'-DIOL FERROCENEBORONATE, MEASURED AT IRON AND BORON LINES

Amount injected (pmol)		*Molar ratio (P:D)*	*Ratio of peak areas (P:D)*	
Pinacol ferrocene boronate (P)	*Dicyclohexyl-1,1'-ferroceneboronate (D)*		*Fe Line*	*B Line*
92.0	126	0.730	0.716	0.740
30.7	42.0	0.730	0.782	
16.7	22.9	0.730	0.878	0.772
5.58	7.64	0.730	0.800	

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CHROMSYMP. 1517

ISOCRATIC ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF UROPORPHYRIN AND COPROPORPHYRIN TYPE II AND IV ISOMERS IN HUMAN URINE

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SUMMARY

Urinary porphyrins of porphyric patients were isolated as their methyl esters by using a simple, modified thin-layer chromatographic system. Existing methods for the isocratic ion-pair high-performance liquid chromatographic separation of uroporphyrin and coproporphyrin isomers were decisively improved by elevating the column temperatures, changing the types of columns used and modifying the eluent compositions. These techniques were applied to the determination of the isomeric distribution of uroporphyrins and coproporphyrins isolated from urines of patients in the acute or latent phase of acute intermittent porphyria. In these urines relatively high contents of the atypical uroporphyrins II (2–5%) and IV (13–19%) were found. The coproporphyrin fractions contained significantly smaller amounts of the atypical isomers II (1–2%) and IV (2–5%), the presence of which was demonstrated for the first time in such urines. Several mechanisms for the formation of the atypical coproporphyrin isomers are discussed. The isocratic ion-pair separation method served also to control the isomeric purity of uroporphyrin specimens of both natural and synthetic origin.

INTRODUCTION

The determination of porphyrins from natural sources has normally been focused on type I and III isomers, as the atypical isomers II and IV were thought to be non-existent in living systems[1]. Substantial improvements in separation and identification techniques for porphyrin isomers now permit detailed analyses for these compounds at trace levels. Isomeric porphyrins can be separated by several chromatographic methods, preferably by high-performance liquid chromatography (HPLC)[2]. Thus, the simultaneous separation of all four isomeric coproporphyrins as free carboxylic acids was performed by using either hydrophobic interaction[3] or ion-pair chromatography[4,5]. Many attempts to separate all four uroporphyrin isomers completely have been reported; however, the separation of uroporphyrins III and IV could not be achieved, even by applying different HPLC techniques[5–10]. Thus, the uroporphyrin isomers must still be converted into the corresponding co-

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proporphyrin isomers by acid-catalysed partial decarboxylation in order to permit a complete analysis of these porphyrins.

Recently, we were able to demonstrate the presence of type II and IV isomers in the total urinary porphyrins of some patients suffering from attacks of acute intermittent porphyria (AIP)[5]. However, the isomeric composition of the individual porphyrin fractions was not elucidated. Formation of the atypical uroporphyrins II and IV, in addition to the normal isomers I and III, was shown to result from non-enzymatic self-condensation of porphobilinogen, which can occur either in the human body or during collection and storage of the urine samples from these patients[5].

In this paper, we report a complete analysis of the isomeric composition of the individual fractions of porphyrins, *e.g.*, uroporphyrins and coproporphyrins, present in the urines of patients with acute or latent forms of AIP. We were especially interested in establishing the isomeric composition of the coproporphyrin fractions, as the formation of these intermediates necessarily involves enzymatic decarboxylation steps under the conditions applied. Application of a modified thin-layer chromatographic (TLC) system allowed a simple separation of the respective urinary uroporphyrin and coproporphyrin fractions. The ratios of the porphyrin isomers were determined by improved isocratic ion-pair HPLC methods, which were based on those described previously[5]. Another aim of this study was to determine the isomeric purity of a series of uroporphyrin specimens originating from both natural and synthetic sources.

EXPERIMENTAL

Reagents

Uroporphyrin I octamethyl ester (original preparation of Fischer and Hofmann[11]), uroporphyrin II octamethyl ester (synthesized by MacDonald and Michl[12]) and a Waldenström uroporphyrin "III" octamethyl ester (original preparation of Waldenström *et al.*[13]) were generous gifts from the Hans-Fischer-Gesellschaft (Munich, F.R.G.). The porphyrin methyl esters were hydrolysed to the free acids by treatment with 6 *M* hydrochloric acid at 25°C for 72 h in the dark. The hydrochloric acid was removed by adsorption of the free porphyrins on Sep-Pak C_{18} cartridges (Waters Assoc., Eschborn, F.R.G.) according to Friedmann and Baldwin[14]. For HPLC analysis the porphyrin free acids were dissolved in a 50 m*M* solution of tetrabutylammonium phosphate in methanol. A statistical mixture of uroporphyrins I (12.5%), II (12.5%), III (50%) and IV (25%) was obtained by the method of Mauzerall[15]. Aqueous phosphate buffer solutions and the ion-pair reagent tetrabutylammonium phosphate were prepared as described elsewhere[5]. Talc was purchased from E. Merck (Darmstadt, F.R.G.).

Apparatus

The HPLC system consisted of a Model 1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.), equipped with a column oven, a Model F-1000 spectrofluorimeter (excitation at 394 nm, emission at 624 nm) (Merck-Hitachi, Darmstadt, F.R.G.) and a Model 1040 diode-array detector (wavelength range 200–590 nm) (Hewlett-Packard).

Urine samples

Urine specimens (24-h) of patients with acute or latent phases of AIP were collected in dark-brown bottles at nearly neutral pH, refrigerated as soon as possible and stored at −20°C[16].

Sample preparation

A 50-ml sample of urine (pH 5–6) was oxidized with a solution of 3 mg of iodine and 6 mg of potassium iodide in 3 ml of water for 10 min at room temperature. After treatment with 5 mg of sodium thiosulphate, the urine was adjusted to pH 3.5 with acetic acid. The porphyrins were adsorbed on 600 mg of talc and washed twice with 20 ml of water. The talc was dried and the porphyrins were dissolved in 10 ml of methanol–concentrated sulphuric acid (9:1, v/v) and kept at 38°C for 40 min. The methanol was removed under vacuum and the residue was dissolved in 10 ml of water. The porphyrin methyl esters were adsorbed on Sep-Pak C_{18} cartridges according to Kennedy *et al.*[17], washed with water and eluted with 10 ml of acetone.

Separation of the porphyrin esters was performed by preparative TLC on silica gel (Kieselgel 60 F, PSC-Fertigplatten, Merck) using toluene–methanol (85:15, v/v) as the solvent. The R_F values of uroporphyrin and coproporphyrin methyl esters were 0.4 and 0.7, respectively. The porphyrin zones were eluted with acetone–methanol (1:1, v/v) and the solvents were removed under vacuum. Hydrolysis of the porphyrin methyl esters with 6 *M* hydrochloric acid and adsorption of the free acids on Sep-Pak C_{18} cartridges were carried out as with the porphyrin methyl ester standards (see above). Uroporphyrins were partially decarboxylated at 180°C to the corresponding coproporphyrins and purified on Sep-Pak C_{18} cartridges, as described elsewhere[5].

Chromatographic conditions

Separation of uroporphyrin isomers. The separations were carried out on LiChrosorb RP-18 (7 μm) columns (250 mm × 4 mm I.D.; Merck), which were protected by a LiChroCART guard cartridge (LiChrosorb RP-18, 5 μm; 4 mm × 4 mm I.D.; Merck). The mobile phase was aqueous phosphate buffer (28 m*M*, pH 5.75)–methanol containing 5 mmol/l of tetrabutylammonium phosphate (pH 7.13) (66:34, v/v). The flow-rate was 1.0 ml/min at a column temperature of 38°C.

Separation of coproporphyrin isomers. The separations were performed on LiChrospher RP-18 (5 μm) columns (125 mm × 4 mm I.D.; Merck) protected by a guard cartridge (see above). The mobile phase was aqueous phosphate buffer (44 m*M*, pH 6.63)–organic phase [methanol–acetonitrile–water (72:21:7, v/v)] containing 8.9 mmol/l of tetrabutylammonium phosphate (pH 7.25) (59:41, v/v). The flow-rate was 1.5 ml/min at a column temperature of 40°C.

RESULTS AND DISCUSSION

Preparation of urine samples

Urine from patients suffering from AIP normally contain large amounts of porphobilinogen, which artificially forms all four uroporphyrin isomers in various ratios under physiological conditions[5]. The non-enzymatic self-condensation of this porphyrin precursor within the human body cannot be avoided, but refrigeration and deep-freezing of the freshly excreted urine largely suppress this reaction during

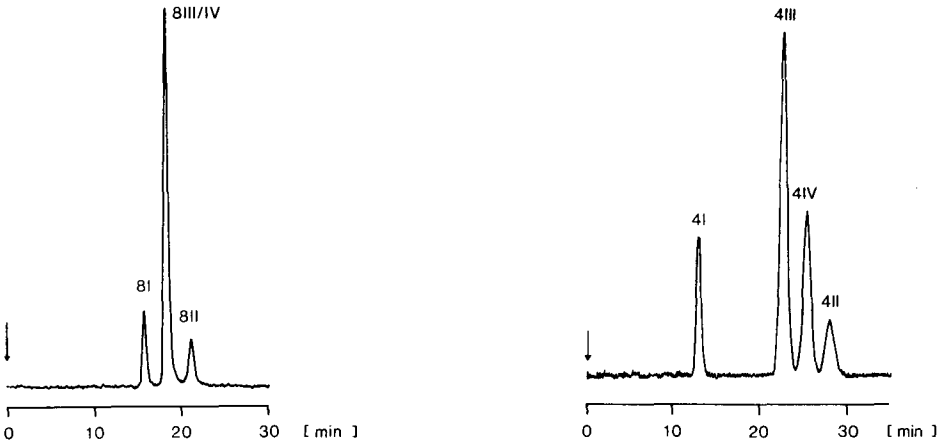

Fig. 1. HPLC separation of a statistical mixture of uroporphyrins I, II and III/IV on a 7-μm LiChrosorb RP-18 column at 38°C. Fluorescence detection.

Fig. 2. HPLC separation of coproporphyrins I, II, III and IV obtained by partial decarboxylation of a statistical mixture of uroporphyrins. Column: 5-μm LiChrospher RP-18 at 40°C. Fluorescence detection.

specimen collection and storage. Furthermore, porphobilinogen can be removed from such urines by treatment with talc, which retains only porphyrins[18].

Porphyric patients excrete considerable amounts of porphyrinogens[19], which are easily isomerized by strong acids[20]. We therefore oxidized the urine samples with iodine at nearly neutral pH according to Mauzerall[20] in order to prevent isomerization of the respective porphyrinogens. Indeed, some isomerization might occur when the oxidation step is carried out under strongly acidic conditions, as applied by Martasek *et al.*[21] and Westerlund *et al.*[22].

Preparative isolation of the individual porphyrin fractions as their methyl esters is normally performed by TLC. We developed a simple solvent system (toluene–methanol) for the TLC separation of uroporphyrin and coproporphyrin methyl esters, thus avoiding the highly toxic benzene present in other solvent systems[23].

Isocratic HPLC separation of uroporphyrin and coproporphyrin isomers

We have previously performed the ion-pair HPLC separation of uroporphyrin isomers under isocratic conditions on a 7-μm LiChrosorb RP-18 column at room temperature[5]. When we now increased the column temperature to 38°C and slightly modified the composition of the mobile phase, *e.g.*, the ionic strength and pH of the aqueous phosphate buffer and the concentration of the ion-pair reagent tetrabutylammonium phosphate in the organic modifier methanol, a sharp reduction in the retention times of all uroporphyrin isomers resulted without any loss of chromatographic resolution (Fig. 1). Using these conditions, the separation of uroporphyrins I, II and III/IV was possible within *ca.* 20 min. However, the isomers III and IV could not be resolved by the eluent system applied, even when the retention time was prolonged to more than 1 h.

The simultaneous ion-pair separation of coproporphyrins I–IV was formerly carried out on a 7-μm LiChrosorb RP-18 column at 38°C[5]. Application of a 5-μm

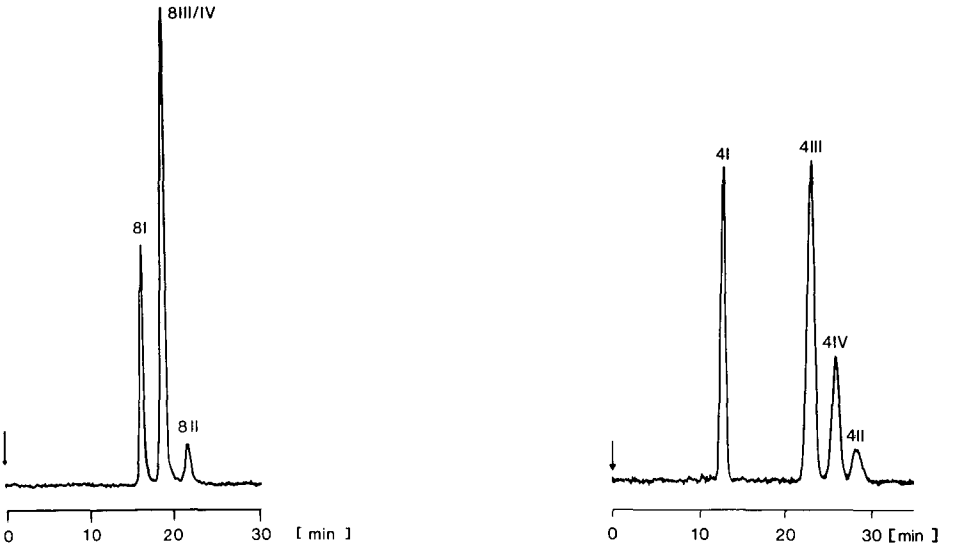

Fig. 3. HPLC separation of urinary uroporphyrins I, II and III/IV isolated from a patient with acute intermittent porphyria (case 1). Conditions as in Fig. 1.

Fig. 4. HPLC separation of coproporphyrins I, II, III and IV obtained after partial decarboxylation of the respective urinary uroporphyrins from a patient with acute intermittent porphyria (case 1). Conditions as in Fig. 2.

LiChrospher RP-18 column at 40°C, together with some modifications of the mobile phase, markedly improved the selectivity, especially for the separation of the isomers II, III and IV (Fig. 2).

HPLC determination of urinary uroporphyrins

The urinary uroporphyrin fractions of four patients (females, 31–40 years old) suffering from acute attacks of AIP were isolated by TLC as their methyl esters. Isocratic ion-pair HPLC analysis of the uroporphyrin free carboxylic acids yielded fairly clean chromatograms, containing only the uroporphyrin isomers I, II and III/IV (Fig. 3). No additional peaks could be detected, in contrast to the chromatograms that we had obtained previously by extraction of urine on talc without the TLC purification step[5]. Partial decarboxylation of the uroporphyrins to the corresponding coproporphyrins and isocratic ion-pair HPLC separation allowed a complete analysis of the isomeric composition originally present in the uroporphyrin fraction (Fig. 4). Table I shows the isomeric distribution of the uroporphyrin fractions from four patients with AIP, obtained by this method. The composition of the resulting coproporphyrin isomers correlated satisfactorily with that of the original uroporphyrin isomers (Table I). In these urines, we found 2–5% of the atypical isomer II and 13–19% of the atypical isomer IV. Thus, we were able to confirm the presence of uroporphyrins II and IV in urines of AIP patients, which we recently described[5]. Owing the mostly non-enzymatic formation of the uroporphyrins from porphobilinogen in AIP patients, the respective isomeric composition depends largely on the retention time and pH of the urine within

TABLE I

ISOMERIC COMPOSITION (%) OF URINARY UROPORPHYRINS (U) FROM FOUR CASES OF ACUTE INTERMITTENT PORPHYRIA

Coproporphyrins (C) were obtained after partial decarboxylation.

Case	*U I*	*U II*	*U III/IV*	*C I*	*C II*	*C III*	*C IV*
1	28.9	5.4	65.7	28.5	4.9	47.8	18.8
2	45.3	2.7	52.0	46.3	3.3	35.9	14.5
3	38.7	3.3	57.9	39.3	3.0	44.0	13.7
4	31.8	3.8	64.4	32.4	3.3	46.1	18.2

the human body. Therefore, the differing ratios of the uroporphyrin isomers (Table I) cannot be used for additional characterization of the individual AIP cases. In these patients, the percentages of the isomers II and IV are comparable to those formed by chemical condensation of porphobilinogen under physiological conditions[5]. In urine samples from control subjects, we observed 80 ± 10% of uroporphyrin I and 20 ± 10% of uroporphyrin III, whereas the atypical isomers II and IV could not be detected until now.

Doss and Schermuly[24] determined the isomeric distribution of all types of porphyrins in the series from uroporphyrin to coproporphyrin in the urine of patients with AIP. In these urine samples only the isomers I and III could be detected. However, the TLC method[25] used by these workers permitted only the separation of the normal isomers I and III, and the questionable presence of the non-typical isomers was not investigated.

HPLC determination of urinary coproporphyrins

The isomeric composition of the urinary coproporphyrin fractions, isolated by TLC, was determined by isocratic ion-pair HPLC in four cases of acute attacks of AIP (cases 2, 4, 5 and 6; females, 31–45 years old; Table II) and two cases in the latent phase of AIP (cases 7 and 8; females, 34 and 42 years old; Table II). Again, clean chromatograms were obtained by this method (Fig. 5).

These urines surprisingly contained the atypical coproporphyrins II and IV, just as found in the uroporphyrin fractions. Positive identification of these unexpected

TABLE II

ISOMERIC COMPOSITION (%) OF URINARY COPROPORPHYRINS (C) FROM SIX CASES OF ACUTE INTERMITTENT PORPHYRIA

Case	*C I*	*C II*	*C III*	*C IV*
2	12.9	1.3	83.2	2.6
4	10.1	1.6	84.6	3.7
5	19.8	1.5	75.3	3.4
6	9.0	0.9	88.3	1.8
7	9.9	2.1	83.0	5.0
8	11.3	2.2	82.1	4.4

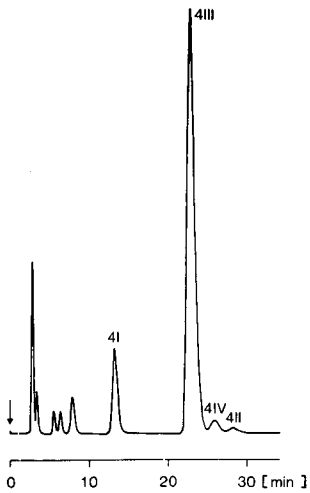

Fig. 5. HPLC separation of urinary coproporphyrins I, II, III and IV isolated from a patient with acute intermittent porphyria (case 2). Conditions as in Fig. 2.

compounds was performed by chromatography after addition of authentic substances and by on-line diode-array detection of their absorption spectra in the wavelength range 300–590 nm. Fig. 6 shows the absorption spectra of the individual coproporphyrin isomers which were overlayed for comparison. The spectrum of isomer IV shows exactly the same curve shape as that of the normal isomers I and III. Isomer II shows a slightly different curve shape at wavelengths above 530 nm. This deviation is caused by the low concentration of isomer II in the urine sample, resulting in an absorption below 0.0005 a.u.f.s. Application of these enhanced separation and

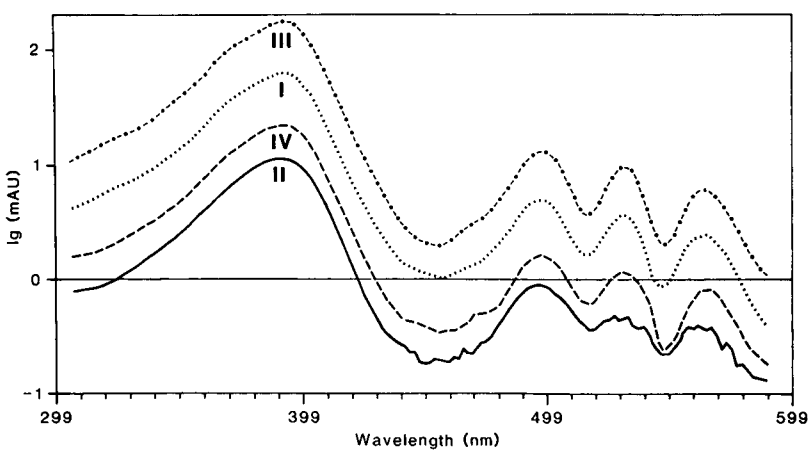

Fig. 6. Absorption spectra of the urinary coproporphyrins I, II, III and IV isolated from a patient with acute intermittent porphyria (case 2). The spectra were recorded by on-line diode-array detection (300–590 nm) and overlayed for comparison.

identification techniques has enabled us to prove the presence of trace amounts of the atypical coproporphyrin isomers II and IV for the first time in such urines. The isomeric proportions of the urinary coproporphyrins of six AIP cases are summarized in Table II. The percentages of the isomers II and IV are significantly lower than those of the atypical uroporphyrins, because we found only 1–2% of isomer II and 2–5% of isomer IV. On the other hand, the ratios of the isomer III are markedly increased and those of the isomer I are decreased in comparison with the ratios of the respective uroporphyrin isomers. Owing to the small number of cases investigated so far, the diagnostic importance of these results still has to be proved by additional data. Urine samples from healthy persons contained the normal coproporphyrin isomers I (20 ± 10%) and III (80 ± 10%) in concentrations similar to those in the AIP patients. The atypical isomers II and IV were found only occasionally in these urines owing to their low concentration. The reference values for normal urines have to be established.

Several mechanisms can be envisioned for the formation of the non-typical coproporphyrin isomers: (1) enzymatic decarboxylation of the respective uroporphyrinogens, formed non-enzymatically from porphobilinogen within the human body; (2) enzymatic decarboxylation of uroporphyrinogens outside the human body by cellular enzymes; this mechanism can be rejected, as only one of the urines investigated contained leukocytes and erythrocytes; (3) chemical isomerization of coproporphyrinogens inside the human body. The last hypothesis is supported by the results of preliminary experiments in which we observed a measurable non-enzymatic rearrangement of coproporphyrinogen III to the other isomers under physiological conditions[26].

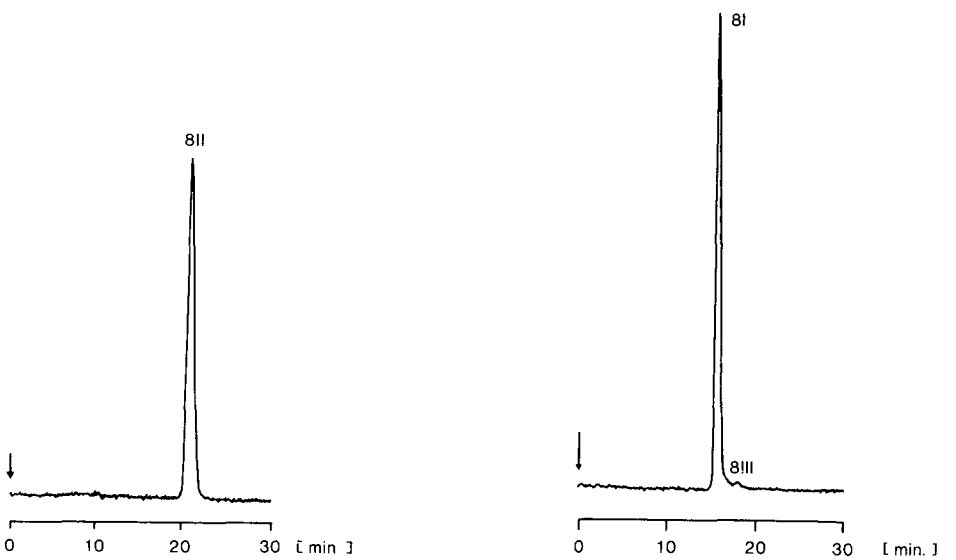

Fig. 7. HPLC analysis of a uroporphyrin II specimen synthesized by MacDonald and Michl[12]. Conditions as in Fig. 1.

Fig. 8. HPLC analysis of a natural uroporphyrin I specimen prepared by Fischer and Hofmann[11]. Conditions as in Fig. 1.

HPLC analysis of uroporphyrin specimens

Isocratic ion-pair separation was applied to check the isomeric purity of a series of uroporphyrin specimens of synthetic or natural origin. The highest isomeric purity was found for a uroporphyrin II specimen synthesized by MacDonald and Michl[12] via the pyrromethene method. The chromatogram in Fig. 7 demonstrates the excellent purity of this specimen, which contained exclusively the isomer II. Lower carboxylated porphyrins were absent, as demonstrated by applying our gradient elution technique[4]. HPLC analysis of a natural uroporphyrin I preparation is shown in Fig. 8. This specimen was isolated and purified by Fischer and Hofmann[11] from the excreta of the famous porphyria case Petry, who suffered from congenital erythropoietic porphyria. The content of isomer I was 99%; only 1% of isomer III was found in this specimen.

We also analysed a so-called Waldenström porphyrin, which had been considered earlier to be uroporphyrin III. The Waldenström porphyrins had been obtained by different isolation procedures from urines of patients in the acute phase of AIP[13]. The nature of these porphyrins has been studied intensively by Nicholas and Rimington[27] with the aid of paper chromatography, Debye–Scherrer X-ray photography and melting-point curves. On the basis of their results, they suggested that the Waldenström uroporphyrins are a mixture of *ca.* 75% of isomer III and 25% of isomer I. HPLC analysis of such a uroporphyrin, originally prepared by Waldenström *et al.*[13], now revealed the presence of isomer I (21%), isomer II (9%) and isomers III/IV (70%) (Fig. 9). Partial decarboxylation to the corresponding coproporphyrins gave 20% of isomer I, 8% of isomer II, 53% of isomer III and 19% of isomer IV. Owing to the presence of substantial amounts of the atypical isomers II and IV in Waldenström's uroporphyrin the mostly non-enzymatic formation of such porphyrins, recognized previously as chemical artifacts by Cookson and Rimington[28], is confirmed by the findings in our study. In addition, we checked the isomeric purity of a series of commercially available uroporphyrin standards. Most of them met the specified

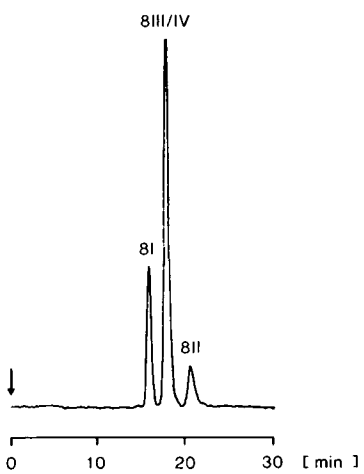

Fig. 9. HPLC analysis of a Waldenström uroporphyrin "III" specimen prepared by Waldenström *et al.*[13]. Conditions as in Fig. 1.

isomeric composition, but in some instances we observed considerable contamination with other isomers, amounting to nearly 50%.

CONCLUSION

Improved isocratic ion-pair HPLC methods are suitable for the determination of the atypical porphyrin isomers II and IV in the urine of patients suffering from AIP. The presence of uroporphyrin isomers II and IV in such urine was confirmed, and the occurrence of the coproporphyrin isomers II and IV was demonstrated for the first time. Formation of the latter is probably due to non-enzymatic isomerization at the porphyrinogen level within the human body.

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METHOD FOR ANALYSIS OF DILUTE VAPOURS IN FLUE GASES AND WORKING ATMOSPHERES

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SUMMARY

Present and forthcoming legislation to control the amount of organic substances emitted in flue gases or present in working atmospheres (*e.g.* laboratories) has required the development of methods for identifying and quantifying parts-per-billion ($1:10^9$) concentrations of a wide range of substances of low and high volatility. A method is described which is applicable to all organic vapours with the exception of very-low-mass compounds. Air or gases to be analysed are passed through an absorption train, containing Tenax GC, the adsorbed organics are desorbed by heating, concentrated into a narrow band, and directly submitted to gas chromatography–mass spectrometry on a Hewlett-Packard 5970B mass selective detector. Substances ranging typically from methyl methacrylate through monoterpenes and plasticizers to nitro- and halobenzenes have been determined as $\mu g/m^3$ in the atmosphere or flue gases.

INTRODUCTION

The increasing use of volatile organic substances in industry and the increasing concern about unnatural substances in the atmosphere have prompted the exploration of methods to measure them and legislation to control their release. In F.R.G., stringent regulations have been made for the concentrations of organic substances permitted in the air of workplaces and in flue emissions[1]. Similar controls will soon be adopted by the European Commission. A directive of the European Economic Community has already been published on combating air pollution, and this applies, among others, to the ceramic industry, for the manufacture of coarse ceramics, refractory, facing and floor bricks, stoneware pipes, and roof tiles. The fine-ceramic industry makes use of a great variety of solvents, adhesives, colour and transfer media, particularly in the application of decoration to pottery and china. When the ceramics are fired in the kiln, these materials are pyrolysed, volatilized, and emitted in the flue gases.

Methods for the analysis of organic substances in the flue gases must therefore be devised. Any method must fulfil at least three requirements. First, it must be as sensitive as possible. Because of the high flow velocities encountered, the volume concentrations may be quite low, and concentration of the contaminants will be necessary. Secondly, the method must be able to detect and quantify a wide range of

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substances, in contrast to the specific tests already available (*e.g.*, for vinyl chloride and isocyanates) in other industries. Thirdly, the equipment must be relatively cheap and robust. Sampling equipment may have to be carried to difficultly accessible locations, and many samples may have to be taken because of varying work conditions, raw materials, and types of kiln.

A method is described here which can fulfil these requirements. The organic substances are concentrated by adsorption of flue gases on a polymeric adsorbent, which is then taken to the laboratory. The volatiles are determined by desorbing them directly into a linked gas chromatograph–mass spectrometer.

MATERIALS AND METHODS

Glass tubes (400 mm × 4 mm I.D.) were packed at one end with a column of Tenax GC (130 mm long, 0.35 g, 35–60 mesh) (Enka, Arnhem, The Netherlands), held in place with silanized glass-wool plugs. The packed end of the adsorption tube was connected to a short length of glass tube, containing a platinum resistance thermometer connected to a digital read-out. This tube was connected to a battery-driven, portable diaphragm pump (Model 224-43XR; SKC, Wimborne, U.K.), capable of drawing air through the adsorbent at up to 600 ml/min. This was, in turn, connected through a drying tube to a Singer total flow-meter (International Gas Apparatus, Camberley, U.K.).

For sampling, the empty end of the adsorption tube was inserted through a hole in the flue so that the open end was near the centre of the flue but the Tenax column remained outside the flue and its insulation, if necessary by using an empty extension glass tube. The pump was switched on, and the time, total volume and temperature were recorded. A 10- or 15-l volume of gas was sampled in this way. The adsorption tubes were capped for transport to the laboratory.

The organic vapours were desorbed thermally by placing the adsorption tube inside an electrically heated tubular oven, with a controllable range of 25°C to 350°C. The adsorption tube was heated to 250°C for 20 min while a stream of helium gas (15 ml/min) was passed through it. The outlet was connected to a glass-lined steel tube (SGE, Milton Keynes, U.K.), bent to form a U-tube and cooled in that portion by immersion in liquid nitrogen in a Dewar flask. From the U-tube the glass-lined metal tube ran through a hole in the injection septum, terminating in the heated zone of the injection block. When all the volatiles had been desorbed from the Tenax and collected in the U-tube, the glass-lined metal tube was flash-heated (less than 10 s) to 250°C by passing a direct current through the metal covering, and the plug of organic vapours was swept on to the chromatographic column. Before re-use, the adsorbent was conditioned at 250°C for 3–6 h under a flow of nitrogen.

Separation was performed on a fused-silica capillary column (12 m × 0.2 mm I.D.), coated with HP-1 (cross-linked methyl silicone gum) of 0.33-μm film thickness in a 5890 gas chromatograph (Hewlett-Packard, Bracknell, U.K.), directly coupled to a HP Mass-Selective Detector 5970B with a HP 59970 Chem Station data system. The chromatographic separation was conducted at 30°C for 2 min, then programmed to 160°C at 3°C/min. Identification of the compounds was made from their mass spectra with the aid of the spectrum library. It was checked by injecting known amounts of authentic specimens and correlating retention time and mass spectrum.

Breakthrough volumes (V_R) were measured in two ways, by connecting a trap directly to a flame detector[2,3] or by using two traps in tandem[2,4]. Using the flame detector method, which was found more convenient, nitrogen was passed through columns of Tenax of the same dimensions as the absorbent beds at flow-rates between 150 ml/min and 200 ml/min and temperatures from 150°C to 70°C. Retention volumes were calculated as described by Poole and Schuette[5].

RESULTS AND DISCUSSION

Adsorption

Tenax GC (surface area *ca.* 20 m^2/g) is a porous polymer, based on 2,6-diphenyl-*p*-phenylene oxide. At the start of this work, Tenax GC was used. Later, a grade specifically for adsorption was introduced, called Tenax TA. A survey of the literature shows it to be the most suitable material for adsorption of organic vapours. Its advantages are that it does not absorb water, it is stable at high temperatures (350°C), substances can be eluted from it readily by heating, and it is relatively unselective for compound types. Its one important limitation is that it does not retain very small molecules, so that the method described automatically excludes the collection of methanol, formaldehyde, acetic acid, acetaldehyde, etc. In addition, these substances give very poor response to a flame-ionization detector, and require special methods. We have described two very sensitive methods for determining lower-mass carbonyl compounds[6,7]. The substances released in fine-ceramic firing are of higher molecular mass, and Tenax is a suitable adsorbent.

A reversible adsorbent, such as Tenax, acts like a chromatographic column when gas is passed through it. It is therefore necessary to be sure that the substances are completely retained during the sampling period. A safe sampling volume or breakthrough volume can be defined as the volume of air containing a particular organic contaminant that can be sampled without a significant amount of contaminants being lost by elution from the adsorbent. Some authors draw a distinction between retention volume (which is slightly greater) and breakthrough volume or safe sampling volume[8]. Values for a number of simple compounds have been recorded[8–10]. Because these values may vary with the size and shape of the adsorbent bed, flow-rate, concentration, etc., we have carried out some experiments to check the breakthrough values of the least-retained compounds met in our analyses, under conditions similar to those we were using. The flow-rates used in determining V_R were only half of those used in actual sampling, but it has been shown[9] for Tenax that V_R values are essentially independent of flow-rate between 100 ml/min and 1000 ml/min.

Plots of log V_R against $1/T$ K (T = temperature), measured at elevated temperatures, gave straight lines, from which values at ambient temperatures could be extrapolated. Some representative values are given in Table I for three substances frequently encountered in sampling. It can be seen that breakthrough volumes are highly dependent upon temperature, and that terpenes have very low breakthrough volumes[11], but these are strongly dependent upon concentration. Riba *et al.*[11] showed that they increase strongly for concentrations less than 5 ppm (28 mg/m^3) and, a breakthrough volume of 12 l/g is recorded for α- and β-pinene at 100 ppb[12] or 0.55 mg/m^3.

The temperature of the adsorbent during sampling is clearly critical. In practice,

TABLE I

BREAKTHROUGH VOLUMES OF SOME REPRESENTATIVE LOW-RETENTION SUBSTANCES IN THE FLUE GASES FROM CERAMIC KILNS

Column, 0.35 g Tenax.

Substance	V_R *(l)*		
	20°C	*35°C*	*250°C*
Methyl methacrylate	42	12.6	$2 \cdot 10^{-4}$
Butyl methacrylate	1400	300	$1 \cdot 10^{-3}$
α-Pinene	1.3[a]	0.12	[b]

[a] At 20 ppm (from ref. 11).
[b] Unretained.

although flue gases are considerably diluted with ambient air drawn into the flue by fans, the temperature in the flue can be as high as 300°C. Preliminary experiments showed that the concentration of pollutants was not critical. The values found were 10–100 times the concentration of organic pollutants in air (Table II)[13]. There was therefore no fear of overloading the adsorbent, sampling could be done in a reasonable time, and to seek much greater sensitivity would have been pointless. Flow-rates allow considerable latitude and were chosen as a compromise between short sampling time and maintaining a low temperature.

Typical total-ion chromatograms are shown in Figs. 1 and 2. The levels of these substances are well below the limits in present legislation and are not a serious contribution to atmospheric organic contaminants. Typical values obtained for a number of samples taken at different times from a kiln, where transfer-decorated pottery was fired, are given in Table III. Further work is in progress on other systems, where flue gases and combustion gases are mixed and can contain soot and large amounts of water vapour.

Legislation in Britain will soon be extended to control of substances hazardous to health in laboratory atmospheres, and it will make provision for controls necessary as the result of the discovery of hitherto unsuspected substances. From our preliminary studies, this method is highly suitable for monitoring of research laboratories where low levels of toxic and constantly varying organic contaminants may be encountered.

TABLE II

RANGE OF SOME MAJOR SUBSTANCES DETERMINED IN THE FLUE GASES OF DECORATIVE CERAMIC KILNS

Concentration and total amount vented to the atmosphere are given. Atmospheric values are reduced to a temperature of 0°C.

Compound	*Concn. (μg/m³)*	*Total amount (mg/h)*
Methyl methacrylate	57–1.6	164–10
Butyl methacrylate	151–1.2	450–5

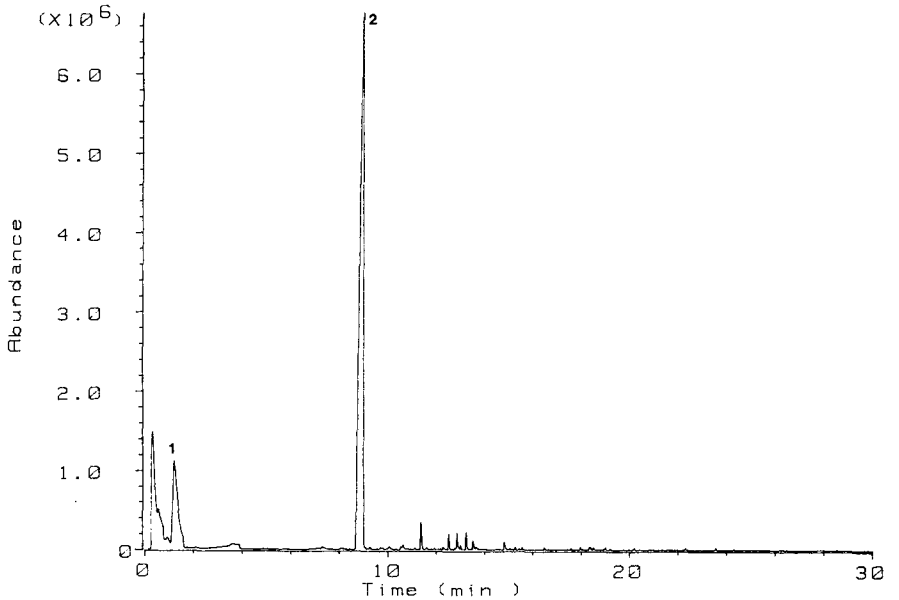

Fig. 1. Total-ion chromatogram obtained from flue gases of kiln firing ceramics decorated with transfers. Peaks: 1 = methyl methacrylate; 2 = butyl methacrylate. Broadening of the methyl methacrylate peak is caused by the "vacuum effect" of having a capillary column directly coupled to a mass spectrometer[14].

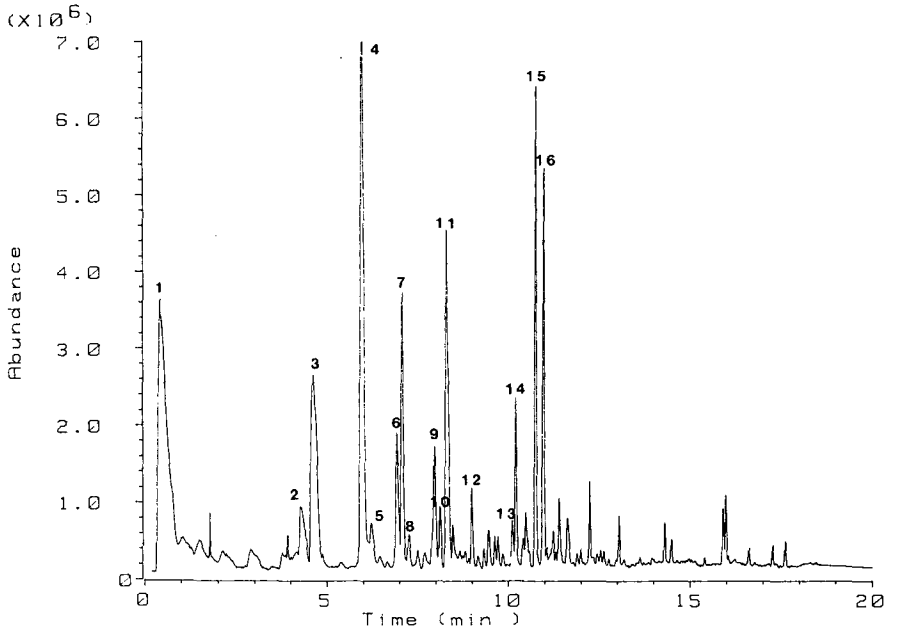

Fig. 2. Total-ion chromatogram obtained from flue gases of kiln firing hand-decorated ceramics. Identified substances: 1 = CO_2; 2 = cyclohexanone; 3 = 2-methylcyclopentanol; 4 = α-pinene; 5 = camphene; 6 = β-pinene; 7 = butyl methacrylate; 8 = toluene; 9 = dichlorobenzene; 10 = *p*-cymene; 11 = cineole (eucalyptol) and limonene; 12 = *o*-cymene and nitrobenzene; 13 = trimethylcyclohexenone; 14 = carene; 15 = camphor; 16 = nitrotoluene. Early peaks are broadened by the "vacuum effect"[14].

TABLE III

RANGE OF VALUES FOUND AT DIFFERENT SAMPLE VOLUMES FOR THE MAJOR ORGANIC CONTAMINANTS IN A SINGLE KILN

Results are given in concentration ($\mu g/m^3$) and total material vented to the atmosphere (mg/h at 0°C). Substances identified as major organic contaminants in Briesco kiln vent.

	Sample size (l) at room temp.	*Sampling temperature (°C)*	*Methyl methacrylate*		*Butyl methacrylate*	
			$\mu g/m^3$	*mg/h*	$\mu g/m^3$	*mg/h*
Sample 1	5	26	12.3	37	58.1	170
Sample 2	5	26	6.1	17	23.2	70
Sample 3	5	26	32.7	92	151	430
Sample 4	10	25	45.0	130	116	337
Sample 5	10	27	20.5	55	110	322
Sample 6	10	25	20.5	58	98.7	285
Sample 7	15	25	57.2	164	104	308
Sample 8	15	25	32.7	92	69.7	205
Sample 9	20	26	20.5	55	134	386
Sample 10	20	26	16.3	49	63.9	178

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DETERMINATION OF THE TOTAL AMOUNT SULFUR IN PETROLEUM FRACTIONS BY CAPILLARY GAS CHROMATOGRAPHY IN COMBINATION WITH COLD TRAPPING AND A TOTAL SULFUR ANALYZER

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SUMMARY

The distribution of sulfur compounds among fractions of a petroleum liquid has been quantitatively determined by capillary gas chromatographic techniques, including on-column injection, cold-trapping of effluent, and measurement of effective recovery. In this study the amount of sulfur was determined in the gasoline (0–450°F), light cycle oil (450-650°F), and heavy cycle oil (650–1100°F) fractions of a refinery stream liquid. Knowledge of the amount of sulfur in the various fractions of this material can be valuable in determining how to process this material in the refinery. The petroleum liquid was separated by an on-column injection into a fused-silica capillary column, and the sulfur in each of the three cold-trapped fractions was measured by a hydrogen sulfide total sulfur analyzer. A detection limit of 50 ppm was achieved with quantitative recoveries of almost 100%. The sulfur distributions determined for a series of these petroleum liquids were 0.01–0.02 wt.-% in the 0–450°F fraction, 0.13–0.23 wt.-% in the 450–650°F fraction, and 0.50–1.20 wt.-% in the 650–1100°F fraction. The reproducibility for duplicate samples was 3%, and the reproducibility for the same sample with multiple cold-trap separations was also within 3%. This method obviates the complicated calibration step, which is necessary when sulfur-specific detectors, such as the flame-photometric detector and the Hall electrolytic conductivity detector are used.

INTRODUCTION

Sulfur compounds are responsible for problems in storage, blending, and processing of crude petroleum fractions in refinery operations. Typical problems caused by sulfur compounds are catalyst poisoning and deactivation, high hydrogen consumption in processing, and corrosion of equipment[1]. In recent years, the supply of petroleum for refinery feedstocks has gradually shifted towards heavier crudes, containing more sulfur compounds, and the presence of the sulfur in the various refinery streams needs to be known in order to efficient operations and to avoid problems.

In the present study, it was of interest to investigate a series of catalytic cracker products that has been produced from a microconfined catalyst bed unit. An atmos-

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pheric residual desulfurization-treated heavy oil was cracked over a wide variety of artificially deactivated commercial catalytic cracking catalysts. The artificially aged catalysts were impregnated either with metal levels characteristic of refinery A or metal levels characteristic of refinery B. Additionally, two of the test samples were produced with equilibrium cracking catalysts. One important piece of information concerning these materials is how sulfur is distributed in the fractions with various boiling points. It is of value to know this for a catalytic cracker product so that it can best be further processed in the refinery. Also, the sulfur distribution will provide useful information on whether the particular catalyst preferentially concentrates sulfur compounds in any of the fractions. Thus, analytical procedure was sought to determine the sulfur distribution in petroleum liquids, such as these catalytic cracker products.

A number of gas chromatographic (GC) methods have been developed for the quantitative analysis of sulfur compounds in various matrices through the use of specific sulfur detectors. Among available sulfur detectors, both the flame-photometric detector (FPD) and the Hall electrolytic conductivity detector (HECD) have enjoyed popularity in interfacing with GC columns[2–5]. However, existing GC methods, associated with these detectors, were found to be unsuitable for quantitating the more than hundred sulfur compounds that are present in a catalytic cracker products, due to several drawbacks of sulfur-selective detectors. The quantitative analysis of sulfur compounds which are present over a wide boiling range (0°F to 1100°F) is very difficult to perform with specific sulfur GC detectors, such as the FPD or the HECD due to the non-linearity of the FPD and the unstable character of the HECD[6].

When a FPD is used, each sulfur compound needs to be individually calibrated, because this detector is not linear and its response varies among compounds. The maximum operating temperature for a detector is generally about 250°C which is well below the oven temperature of 350°C required to elute all sulfur compounds. Also, a quenching effect, which reduces the intensity of response to sulfur compounds, has frequently been found when hydrocarbons are eluted together with sulfur compounds[5].

A HECD may eliminate some of the problems associated with the operation of a FPD. Extensive calibration is not necessary with a HECD because it is a linear detector. The detector temperature is easily increased to 350°C without any problems[6]. Unfortunately, poor reproducibility has been a problem when a HECD was used with the wide temperature programming which is required in simulated distillation type GC analysis of crude oils and other heavy hydrocarbon feedstocks[7]. Recently, Bradley and Schiller[8] reported a method for assessing the sulfur distribution in heavy oils by means of a pyrolyzer and a FPD. Although this method utilized the FPD in a manner that eliminated several problems, such as the detector temperature limit and the need of extensive calibrations for individual sulfur compounds, it still required a time-consuming calibration of sulfur dioxide. Thus, there is still a need for a simple and reliable method of determining the distribution of sulfur compounds in petroleum.

Knowledge of the amount of sulfur present in each of three boiling point fractions of the catalytic cracker products (gasoline at 0–450°F, light cycle oil at 450–650°F, and heavy cycle oil at 650–1100°F) would provide the type of information valuable in processing these materials and in evaluating the catalysts. This report

describes the development of a new method for the determination of total sulfur compounds in each of these fractions in petroleum liquids, such as the catalytic cracker products.

This method combines on-column injection with cryogenic trapping of effluents in capillary tubing. A hydrogen sulfide-type total sulfur analyzer was used to measure the sulfur compounds recovered. The Houston–Atlas hydrogen sulfide analyzer has been found to be reliable and sensitive, without interference from nitrogen and halogens[9]. The on-column injection into a fused-silica capillary column is becoming increasingly popular for quantitation[10]. The use of a fused-silica capillary column in our work had the advantage over a packed column of reducing the adsorption of sulfur compounds.

EXPERIMENTAL

Simulated distillation capillary GC with a cold-trap loop

The GC equipment utilized for this work was a Varian Model 3700 (Walnut Creek, CA, U.S.A.), equipped with a FID and a dual flame photometric detector. An additional on-column injector, Model OCI-2 (SGE, Austin, TX, U.S.A.) was installed on the GC oven wall, and the carrier gas, helium, for an unused packed-column injection port was diverted into the on-column injector. A fused-silica capillary column, DB-5 25 m × 0.32 mm I.D. with 1-μm film thickness (J&W, Folsom, CA, U.S.A.) was connected to the on-column injector. The outlet end of the capillary column was connected to a cross (Valco, Houston, TX, U.S.A.) which diverts the column effluents into three capillary trap loops, made of 0.031-in. stainless-steel tubing.

The analytical column, the cross, and a portion of the sample trap loop were located inside the GC oven, while one third of the trap loop which was located outside the GC oven was submerged in liquid nitrogen. Two cold-traps were connected in series from each of the three cold-trap loops. The first trap loop (which will trap the low-boiling compounds along with the solvent) was prepared by inserting a stainless-steel tube, 10 cm × 0.1 in. I.D., in the middle of a capillary tube in order to prevent clogging by a large amount of solvent being eluted. The 0.31-in. capillary stainless-steel tube was connected with a 0.1-in. stainless-steel tube by means of a capillary butt connector and double-tapered ferrule (Supelco, Bellefonte, PA, U.S.A.).

The sample was first diluted with ten volumes of toluene. A 3-μl sample was then introduced, using an on-column injection syringe with a fused-silica needle (12 cm × 0.17 mm I.D.). The oven temperature was programmed from 30°C to 350°C at 8°C/min. The first fraction (0–450°F) was eluted when the oven temperature reached 140°C, the second fraction (450–650°F) when it reached 220°C, and the third fraction (650–1100°F) when it reached 340°C. The oven temperature for each fraction was calibrated using the boiling points of hydrocarbons and sulfur compounds with both FID and FPD. While the first fraction was being collected on the first trap-loop, the other two trap-loops were plugged with septa.

Sulfur compounds (along with other trapped components) were recovered by passing approximately 400 μl of toluene (a rinse volume that was several times the volume of the loop in order to recover all material efficiently through the capillary trap-loop). The glass injection reservoir, containing the toluene, was pressurized to

1 lb./in.^{2} with nitrogen, and the flow-rate of toluene was controlled with a needle valve so as not to exceed 0.1 ml/min.

Measurement of total sulfur content of separated fractions

A Houston–Atlas total sulfur analyzer, Model 856 Tracor Atlas, Houston, TX, U.S.A.) was used to measure the total sulfur content of each fraction recovered from the cold-traps. This instrument consists of a total sulfur hydrogenator (Model 856) and a total hydrogen sulfide analyzer (Model 825R-D). Approximately, 80 μl of the toluene solution from the cold-trap was injected into the Model 856 pyrolyzer at 1250°C. The analyzer was operated at a hydrogen flow-rate of 30 ml/min and a sample injection rate of 5 ml/min. For the injection of a large amount of sample at a constant rate, a micro-jet automatic injector (Tracor Atlas, Model 1001) was used. A 0.4-μg/ml solution of butyl sulfide in toluene was used to calibrate the first and second fractions. A dodecyl sulfide (boiling point, 485°C) solution of 0.4 μg/ml was used to calibrate the third fraction.

Recovery of sulfur compounds

In order to determine the recovery of sulfur compounds by the cold-trap and Houston–Atlas analyzer, standard solutions of sulfur in toluene at concentrations of 10–100 ng/μl were prepared and analyzed. Thirteen groups of sulfur compounds with boiling points ranging from 289 to 657°F were used individually to evaluate the sulfur response for this study. These are shown along with their boiling points in Table I. The fourth column in Table I shows the amount of sulfur known to be introduced into the column as the standard, and the fifth column shows the amount of sulfur recovered. Most of the compounds, except the dibenzothiophenes and some disulfides showed almost 100% recovery (sixth column of Table I).

The seventh column in Table I shows recoveries obtained when the Houston–Atlas hydrogen sulfide analyzer was calibrated in each case with a standard, made from the same compound as that being analyzed, rather than by using the single calibration with the butyl sulfide standard for all compounds. The recoveries then approach 100% in all cases, with particular improvement in the dibenzothiophenes and disulfides. This type of external calibration compensates for hydrogenator efficiency and sulfur detector response to the individual sulfur compounds and results in recoveries of almost 100%. The reason for the improvement when such self-calibration is carried out is seen in Fig. 1, which shows the response of the Houston–Atlas hydrogen sulfide analyzer to a number of sulfur compounds. There is clearly a variation among these compounds, some groups, such as the dibenzothiophenes, showing much lower response than the majority of the others. A low response of this analyzer to dibenzothiophenes has been reported previously by Drushel[9], who showed that an improved response could be obtained by increasing the temperature of a pyrolyzer. It therefore follows that such compounds would show a low recovery when calibrated with a compound like butyl sulfide, which shows a significantly higher response in the Houston–Atlas analyzer. Low Houston–Atlas response and low recoveries in these cases are not due to the boiling points of the compounds, because their boiling points are close to those of others which show high response. The excellent recoveries obtained with self-calibration indicate that components are not being lost or discriminated against in the overall procedure involving GC separation, cold-trapping, and sulfur analysis.

TABLE I

PERCENT SULFUR RECOVERY FOR SULFUR COMPOUND BLEND

Amount of sulfur present and recovered includes all compounds in group.

Compound	*b.p. (°F)*	*Group no.*	*Test no.*	*Calc. (ng)*	*Recovery[a] (ng)*	*Recovery (%)*	*Direct compound recovery[c] (%)*
n-Propyl sulfide	289	1	1	68	70.2	103	100
			2	68	63.0	93	95
n-Pentyl sulfide	527	2	1	82	65	79.2	100
			2	82	62.5	77	96
			3	170	205	122	100
Dibenzothiopene	640	3	1	181	114	63	92
			2	181	132	73	100
Thiophene	183	4	1	60	65	107	100
			2	60	83	138	128
Dodecyl mercaptan	530	5	1	23	21	91	100
			2	46	50	108	100
Multicomponent groups							
Dibenzothiophene	640						
1-Benzothiophene	428	6	1	140	62	44.3	100
2-Ethylthiopene	273		2	140	68	48.5	100
Amyl mercaptan							
Hexyl mercaptan	280	7	1	81.2	93.7	109	115
Heptyl mercaptan	325		2	81.2	94	116	110
Butyl disulfide	448						
Pentyl disulfide	527	8	1	207.9	198.9	96	102
Phenyl disulfide	657		2	207.9	204	98	103
Ethyl sulfide	198						
n-Propyl sulfide	289	9	1	98	111	113	99
n-Butyl sulfide	372		2	98	110	113	99
Dimethyl disulfide	99.2						
Dibutyl disulfide	448	10	1	121.7	104	88	100
Dipropyl disulfide	388		2	121.7	97	80	100
2-Ethyl thiophene	273						
Propyl disulfide	388	11					
Dodecyl mercaptan	530		1	62.2	66.9	107	100
Phenyl sulfide	565		2	62.2	65.9	103	100
Diallyl sulfide	280.4						
Methyl *n*-octyl sulfide	651						
Methyl *n*-nonyl sulfide		12	1	262.2	355	135	98
Heptyl sulfide			2	262.2	251[b]	96	100
Phenyl sulfide	565		3	262.2	257[b]	98	100
Octyl mercaptan	390						
t-Dodecyl mercaptan	509						
t-Decyl mercaptan	442	13					
sec-Amyl mercaptan	234		1	350.7	331	94.3	107
Hexyl mercaptan	306		2	350.7	315.8	91	103

[a] Butyl sulfide (0.4 mg/ml) was used to calibrate the concentration on a Houston–Atlas total sulfur analyzer.

[b] Heptyl sulfide (0.4095 mg/ml) was used to calibrate this concentration.

[c] Direct compound recovery was compared with its own solution instead of butyl sulfide solution.

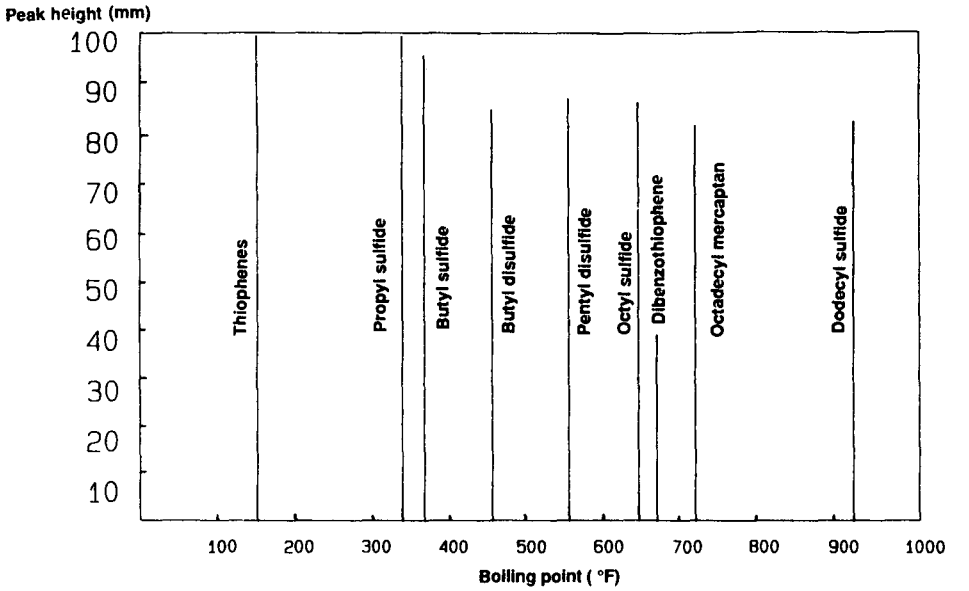

Fig. 1. Different responses of Houston–Atlas total sulfur analyzer to various compounds.

Calibration for individual compounds is time-consuming and is not even possible for samples where the specific sulfur compounds are not known. Examination of the results for the butyl sulfide calibration in Table I shows that overall the recovery for the various compounds is quite good and that acceptable results should be obtainable with this procedure.

The response (peak height) of the Houston–Atlas analyzer was found to be linear with sulfur concentration between 30 and *ca.* 200 ng. The error in measuring peak heights in the analyzer with a signal-to-noise ratio of about 2–8 was estimated to be below 4–5 ng. Most reading fall in the 100–220 ng range, so that the signal-to-noise ratio for these was between 2 and 4%.

RESULTS AND DISCUSSION

Sulfur distributions were determined for eleven catalytic cracker products and one reference liquid product which was not treated, and the results are shown in Table II. The catalytic cracker products were all produced by a microconfined catalyst bed unit which was fed an atmospheric residual desulfurization-treated heavy oil. The catalysts for nine of the samples (1–9) were artificially aged in the laboratory. Five of these (1, 3, 5, 7 and 9) were impregnated with metal levels (1200 ppm Ni, 1800 ppm V and 600 ppm Sb) characteristic of refinery A, and these are designated as (A) in Table II. Four of these (2, 4, 6 and 8) were impregnated with metal levels (2500 ppm Ni, 3500 ppm V and 1200 ppm Sb) characteristic of the refinery B, and these are designated as (B) in Table II. The catalysts impregnated to the refinery A metal levels were used to crack an atmospheric residual desulfurization-treated refinery A crude containing 0.31 wt.-% sulfur. The catalysts that were impregnated to the refinery B

TABLE II

SULFUR DISTRIBUTION IN CAT CRACKER PRODUCTS

Sample [c]	*Catalyst*[d]	*wt.-% Sulfur in fractions*[a]			*wt.-% Hydrocarbon in fractions*[b]		
		F-1 (Gasoline) (0–450°F)	*F-2 (Light cycle oil) (450–650°F)*	*F-3 (Heavy cycle oil) (650–1100°F)*	*F-1 (Gasoline) (0–450°F)*	*F-2 (Light cycle oil) (450–650°F)*	*F-3 (Heavy cycle oil) (650–1100°F)*
1(A)	P	0.01	0.23	0.57	59.17	25.11	15.72
2(B)	C1	0.01	0.15	0.50	62.40	22.00	15.60
3(A)	C2	0.01	0.22	0.83	67.73	22.01	10.28
4(B)	C1	0.01	0.15	0.50	62.40	22.00	15.60
5(A)	C2	0.01	0.26	0.70	62.73	22.01	15.26
6(B)	D1	0.02	0.17	0.78	66.07	24.10	9.83
7(A)	D2	0.01	0.19	1.07	69.21	23.02	7.77
8(B)	E1	0.02	0.13	0.62	61.51	25.56	12.93
9(A)	E2	0.01	0.21	0.92	65.38	24.50	10.12
10	F1	0.02	0.17	1.20	68.29	23.76	7.95
11	F2	0.02	0.22	0.70	63.68	23.80	12.52
12	Ref.liq.prod.	0.03	0.19	0.35	54.12	22.00	23.88

[a] Sulfur content as a percentage of weight in each fraction. wt.-% sulfur $= \dfrac{\text{wt. of sulfur in fraction}}{\text{total wt. in fraction}}$

[b] Percentage of total hydrocarbon sample present in each fraction (determined by simulated distillation analysis).

wt.-% hydrocarbon $= \dfrac{\text{wt. hydrocarbon in fraction}}{\text{total sample weight}}$

[c] A = refinery A; B = refinery B.

[d] Real catalyst name is not shown.

metal levels were used to crack an atmospheric residual desulfurization-treated refinery B fresh feed, containing 0.3 wt.-% sulfur. The remaining two samples (10 and 11) were prepared by using equilibrium catalysts. Sample 12 is a reference liquid product, which is used to calibrate the hydrocarbon boiling point distribution in the simulated distillation test procedure. The first three columns in Table II show the concentration of sulfur in each of the three fractions. This is given as weight percent of the amount of material in the particular fraction, so that the numbers shown represent the sulfur levels that would be present in a stream consisting of that fraction alone. In order to determine the weight percent for each fraction from the amount of sulfur measured in the cold-trap, it was necessary to know the distribution of the hydrocarbon in the sample among the fractions. This was obtained from a simulated distillation GC analysis, and the results are shown in the last three columns of Table II.

The results in Table II show that in the catalytic cracker products, the sulfur levels were 0.01–0.02 wt.-% in the gasoline fraction (0–450°F), 0.13–0.23 wt.-% in the light cycle oil fraction (450–650°F), and 0.50–1.20 wt.-% in the heavy cycle oil fraction (650–1100°F). Samples 2 and 4 were duplicates, as were samples 3 and 5. Comparison of results in Table II for those duplicate sample pairs shows that reproducibility obtained with this technique was good.

We have noted that the sulfur levels measured in the light cycle oil cut (450–650°F) and heavy oil cut (650–1100°F) of the microconfined catalyst bed unit (MCBU) products are consistently *ca.* 1.5 times higher for the products from the experiments made by using a catalyst impregnated to refinery A metals level and a refinery A feedstock than those made by using a catalyst impregnated to refinery B metals levels and a refinery B feedstock. Specifically, we are referring to analyses runs 2 and 3, 4 and 5, 6 and 7, and 8 and 9 for catalyst C, D, and E as shown in Table III. Samples 2 and 4 were identical and treated with the same catalyst as were samples 3 and 5. The wt.-% of sulfur in the fractions were surprisingly reproducible, as shown in Table IV. When the analyses were performed, the analyst did not have any information on these samples. These results proved that the procedure itself is very reproducible and applicable to routine analysis. From the MCBU experiments we know that the experiments on refinery B-metal and feed material resulted in lower conversions and greater selectivities towards hydrogen and coke than those on refinery A-catalyst materials.

TABLE III

FEED PROPERTIES

		Refinery A topped crude	*Refinery B fresh feed*
American Petroleum Institute (API) at 60°F		19.5	22.1
Carbon residual	(wt.-%)	4.7	3.1
Sulfur	(wt.-%)	0.30	0.31
Total nitrogen	(wt.-%)	0.14	0.17
Basic nitrogen	(ppm)	830	658
Saturates	(wt.-%)	57.4	55.0
Aromatics	(wt.-%)	26.3	30.4
Resins	(wt.-%)	11.8	10.6
Asphaltenes	(wt.-%)	4.4	3.4

TABLE IV

WEIGHT PERCENT SULFUR IN HEAVY CYCLE OIL FRACTION

wt.-% sulfur in heavy cycle oil A/B = C 1.58, D 1.37, E 1.48.

Sample [a]	*Catalyst*	*wt.-% sulfur in fraction*
2 (B)	C1	0.50
3(A)	C2	0.83
4(B)	C1	0.50
5(A)	C2	0.70
6(B)	D1	0.78
7(A)	D2	1.07
8(B)	E1	0.62
9(A)	E2	0.92

[a] A = refinery A, B = refinery B.

Since the sulfur from the feeds could turn up in any of three product stream (the liquid product, the gas products, or the coke), the sulfur unaccounted for in the liquid products of the refinery B experiments must have been converted to hydrogen sulfide or deposited as coke. Several explanations of this phenomenon are possible and, most likely, they all contributed to the overall result. One explanation could be that the higher nickel concentrations resulted in dehydrogenation and condensation of heavier sulfur-bearing hydrocarbon components and resulted in the selective concentration of the incremental sulfur in the coke. Another explanation could be that the feedstocks and/or the differences in the feed pretreatment (atmospheric residual desulfurization processing) between the two facilities (refineries A and B) could account for the sulfur-containing fraction of the refinery B feed being more refractory than the sulfur-containing fraction of the refinery A feed, resulting in preferential condensation of the incremental sulfur-bearing material as coke. In any case, we do not have the information necessary to determine why the recovery of sulfur in the liquid products differed in such a consistent manner, and additional experiments would be necessary to confirm these results and determine the reasons for them. What is important to note is that this analysis technique allowed us to observe these differences.

Table V shows the amount of sulfur in each of the samples found by various techniques. The first three columns show the amount of sulfur in nanogram, found in each fraction for a given total sample volume (in 3 μl of diluted sample) by the present GC–cold-trap method, and the fourth column gives the total of the three fractions. In addition to this method, the total amount of sulfur for this same volume of each sample was measured by X-ray fluorescence, and this result is shown in the fifth column. The X-ray fluorescence technique determines the weight percent of sulfur in the sample, and this number can then be converted to nanogram in the injected volume of sample in the GC–cold-trap method. This is the number shown in the fifth column, while the weight percent is shown in parentheses. The sixth column shows the recovery for the GC–cold-trap method relative to the sulfur amount determined by X-ray fluorescence. The last column in Table V shows the amount of elemental sulfur as determined by the polarographic method. The amount of elemental sulfur

TABLE V

SULFUR CONTENT IN CAT CRACKER PRODUCTS BY DIFFERENT TECHNIQUES

Sample	*Catalyst*	*Total sulfur by cold trap (ng)* [a]				*Total sulfur*[b] *by X-ray fluorescence*		*Recovery (%)*	*Elemental sulfur*[c] *by polarographic method*	
		F-1 (Gasoline) (0–450°F)	*F-2 (Light cycle oil) (450–650°F)*	*F-3 (Heavy cycle oil) (650–1100°F)*	*Total*	*ng*	*wt.-% in sample*		*ng*	*wt.-% in sample*
1(A)	P	18.2	152.3	233.1	403.6	369.2	(0.14)	109	3.4	(0.0013)
2(B)	C1	22.0	84.3	203.6	309.9	316.4	(0.12)	97.9	1.8	(0.0006)
3(A)	C2	10.6	124.9	220.4	355.9	394.2	(0.15)	94.3	3	(0.0010)
4(B)	C1	19.5	85.3	203.5	308.3	342.8	(0.23)	90	0.6	(0.0002)
5(A)	C2	24.4	150.3	276.5	451.2	395.5	(0.15)	114	2.4	(0.0008)
6(B)	D1	31.9	103.9	199.9	335.7	263.7	(0.10)	127	3	(0.0010)
7(A)	D2	23.9	111.9	216.6	352.4	369.2	(0.14)	95.5	2.7	(0.0009)
8(B)	E1	25.2	87.7	206.9	319.8	316.4	(0.12)	101	2.7	(0.0009)
9(A)	E2	18.2	136.0	241.2	395.4	395.5	(0.15)	100	3.3	(0.0011)
10	F1	30.5	107.4	249.0	386.9	369.2	(0.14)	104.8	2.1	(0.0007)
11	F2	26.8	134.1	226.8	387.7	316.4	(0.12)	123	3.3	(0.0011)
12	Ref.liq.prod.	36.6	107.0	215.4	359.0	321.0	(0.63)	112	<0.6	(<0.0002)

[a] Samples were diluted with toluene in 1:10 ratio prior to injection. Sample 12 was diluted 1:50. Amount of sulfur shown is that found in 3 μl injected volume of the diluted sample.

[b] X-Ray fluorescence data given result as wt.-% of sulfur in sample. Amount of sulfur shown is that which would be contained in the injected volume of diluted sample for the corresponding GC–cold trap test in preceding columns.

[c] Polarographic data given result as wt.-% of elemental sulfur in sample. Amount of sulfur shown is that which would be contained in the injected volume of diluted sample for the corresponding GC–cold trap test in the preceding columns.

was generally found to be less than or equal to 0.0010 wt.-% in three catalytic cracker products.

The largest discrepancies between the X-ray method and the GC–cold-trap method were 14%, 23%, and 27% (Table V). The remaining nine samples showed agreement within 10%, which is considered reasonable for comparisons between two completely different procedures. The precision of sulfur analyses by X-ray fluorescence is generally about ±5%[11]. Differences may be due to several reasons in the cold-trap method, such as inaccurate injection volumes, unusually high response of the sulfur analyzer, and misuse of the correction factors. The error in the 3-μl volume of sample injected was *ca.* ±0.15 μl. The detector, consisting of a lead acetate tape in the Houston–Atlas analyzer often gives a little different response in repeated use without good conditioning, and this can also introduce some error. All these factors may lead to *ca.* 5% uncertainty in the results.

Excluding the two samples which showed more than 20% difference, the total sulfur contents of catalytic cracker samples obtained by this method differed by about 5% from those obtained by X-ray fluorescence method. This might indicate the accuracy obtainable in the cold-trap method. Thus, it is apparent that the cryogenic trapping of effluents in the capillary tubing and their subsequent recovery was quantitative. Cryogenic trapping in a capillary tubing was originally used in the purge-and-trap method for the analysis of volatile organics. The trapping efficiency is controlled by using a different film thickness when trapping is performed at room temperature[12]. The present method is novel in that it involves the trapping of three fractions in series, cut from column effluents covering a wide boiling range. The use of an inert fused-silica capillary column eliminates the adsorption of polar sulfur compounds that occurs in a packed column. Non-reproducible recovery of some mercaptanes was thought to be due to such adsorption[8]. The recovery for mercaptans in this method was quite reproducible.

The good overall agreement between this procedure and X-ray fluorescence obtained in this study is ascribed to the nature of the present samples. Even though large discrepancies were observed for some individual sulfur components in this method, the overall blend of sulfur compounds present in the samples themselves resulted in a good determination of the total sulfur content. When samples were analyzed in triplicate by this method, the standard deviation for the values determined was *ca.* 3%. These replicate analysis results show that the precision for sulfur determination in fractions of catalytic cracker products by this method is *ca.* 3%.

Most of the sulfur compounds in these catalytic cracker products were identified as benzothiophenes and dibenzothiophenes by mass spectrometric analysis. Dibenzothiophenes begin to appear at 600°F (which is the last part of fraction 2), followed by isomers of alkyl group-substituted dibenzothiophenes (mostly methyl, ethyl, propyl, butyl, and amyl substituted). Benzothiophenes begin to appear at 500°F (the first part of fraction 2). The benzothiophenes consist of several families, substituted by alkyl groups, just like the dibenzothiophenes. Longer alkyl chains, such as the decyl group (which is usually found in crudes) were not detected in the mass spectrometric analysis. Dealkylation is believed to occur during the distillation of crudes. Mercaptans and volatile sulfides constitutes less than 1% of the total sulfur, according to the results of both sodium hydroxide and silver nitrate tests. Heavy sulfides and multiple-ring thiophenes apparently predominate. Disulfides were not

TABLE VI

SULFUR DISTRIBUTION IN A CAT CRACKER PRODUCT BY GC WITH SPECIFIC SULFUR DETECTOR (HECD)

	wt.-% sulfur in fractions (by cold trap)			*wt.-% sulfur in fractions (by HECD specific sulfur detector)*		
Sample 12, ref. liquid product	*F-1 (gasoline) (0–450°F)*	*F-2 (light cycle oil) (450–650°F)*	*F-3 (heavy cycle oil) (650–1100°F)*	*F-1 (gasoline) (0–450°F)*	*F-2 (light cycle oil) (450–650°F)*	*F-3 (heavy cycle oil) (650–1100°F)*
Run 1				0.02	0.16	0.40
Run 2	0.03	0.17	0.32	0.02	0.25	0.33
Run 3	0.04	0.19	0.29	0.01	0.25	0.33
Run 4	0.03	0.18	0.29	0.01	0.18	0.40
Run 5	0.03	0.14	0.24	0.01	0.29	0.30
Run 6				0.02	0.20	0.36
Run 7				0.01	0.22	0.37
Mean	0.03	0.17	0.30	0.01	0.16	0.36
Standard deviation	0.0045	0.021	0.041	0.005	0.045	0.038

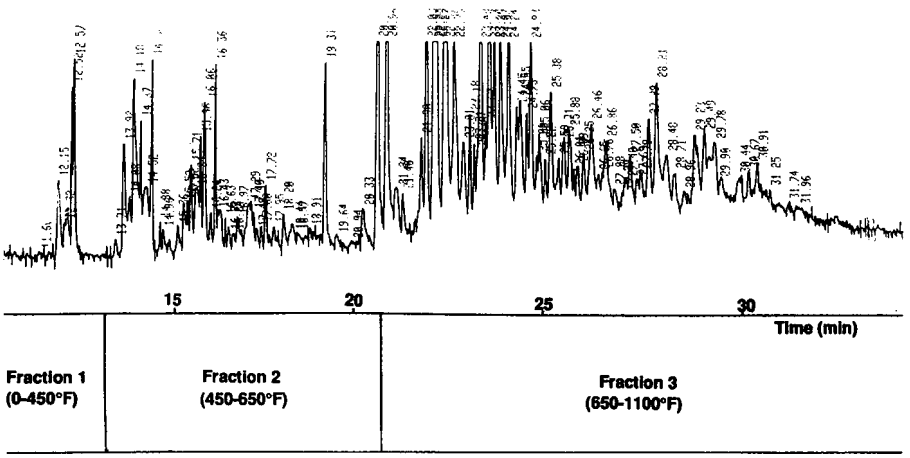

Fig. 2. Chromatogram of a references liquid product. Column, DB-1, 30 m × 0.32 mm I.D.; 1-μm film thickness; carrier gas, helium; detector, HECD at 350°C; temperature programming, 30 to 350°C with 6°C/min; on-column injection; sample size, 1 μl, diluted 1:10 in toluene.

found by mass spectrometry. A number of literature references also indicate that disulfides are not found in gas oils[13].

On-column injection into a fused-silica capillary column was verified in this study as a powerful technique for the quantitation of complex samples, such as the sulfur compounds in these catalytic cracker products. It has previously been reported that column injection modes, such as split or splitless injection, can cause component discrimination so that quantitation is not achieved[14]. Many laboratories have reported that on-column injection is superior to injection through a hot injection port[15]. The former was found to be reliable, rugged, and simple to perform by any person familiar sample trapping techniques.

As a comparison of techniques, sample 12 was analyzed with a simulated distillation gas chromatograph (SIMD), interfaced with a HECD. Results of replicate analyses are shown in Table VI. A chromatogram from one of these analyses is shown in Fig. 2. The sulfur distributions for the three fractions are calculated based upon the sulfer area in each fraction divided by the total sulfur area obtained with the HECD. The standard deviation for this SIMD–HECD method (Table VI) is greater than for the cold-trap method. It is interesting to observe that the mean distribution from seven analyses of fraction 3 is 60.8%, compared to the value of 60.0% for the cold-trap method, shown in Table I. However, the poor reproducibility associated with the SIMD–HECD method appeared to be a problem. The response of the HECD also varies with the type of sulfur compound. This means that quantitation of the different compounds in a sample requires knowledge of the identity of each sulfur component and a calibration for each component. For complex samples, such as these catalytic cracker products, calibration for each component is tedious and time consuming. Moreover, it is impossible to perform a complete calibration, since standards for many sulfur containing compounds are not available. The FPD suffers less from response variation. However, the operating temperature of the FPD is much below

the temperature needed to elute many components of interest in samples, such as these catalytic cracker products, and the use of a column temperature above the detector temperature is undesirable due to problems with condensation in the detector.

Further study is necessary to refine the present method in order to reduce the error and automate the entire procedure or make it semi-automatic by use of a microsampling valve. An alternative to the Houston–Atlas hydrogen sulfide-type total sulfur analyzer could be a sulfur dioxide-type sulfur analyzer, which has been claimed to provide better detection.

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CHROMSYMP. 1556

CONFIRMING CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS WITH STEREOSPECIFIC ENZYMES

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SUMMARY

Identification of optical isomers of amino acids, separated by chiral high-performance liquid chromatography, has proved to be difficult. Despite highly selective separation techniques, identification of peaks based on retention times alone is usually uncertain, particularly in assays of complex biological samples in which interfering compounds are eluted with the peak of interest. We describe here an approach for increasing the certainty of identification of peaks of D- and L-amino acids by the use of stereospecific amino acid oxidase and racemase enzymes. A portion of the sample is first incubated with the enzymes. The amino acids in the treated and untreated portions are then chromatographed and the chromatograms of the samples with and without enzyme incubation are compared. The differences are used to help identify the amino acids.

INTRODUCTION

We have described several effective methods for the high-performance liquid chromatographic (HPLC) separation of optical isomers of amino acids after derivatization with dansyl chloride[1] or with *o*-phthalaldehyde in the presence of N-acetyl-L-cysteine[2]. In applying these techniques for assaying D- and L-pipecolic acids in the urine of patients with an inborn error of amino acid metabolism[3], we noted that these compounds, which were present in low concentrations, were eluted at approximately the same time as other minor components of the mixture and could not be reliably be distinguished from them. Retention time was not specific enough; it helped only to locate the amino acid. Before stereoselective chromatographic procedures were developed, D-, L-specific amino acid oxidases were used to distinguish optical isomers[4,5]. The amino acid oxidases, which react with amino acids either in the D or L series, are only semi-specific. Earlier, the enzymatic procedure was often performed in conjunction with ion-exchange chromatography if more than one amino acid is expected in the mixture. Recently, an entirely different approach for improving the confidence of D and L optical isomer identification involving an optical activity detector was introduced by Reitsma and Young[6,7]. In the work presented here, the enzyme-assisted assay was performed in conjunction with a reversed-phase HPLC procedure that effectively resolves racemic mixtures of most amino acids but is unable to separate all possible amino acid metabolites in biological mixtures.

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EXPERIMENTAL

Reagents

Methanol and acetonitrile, "distilled-in-glass" quality, were bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Amino acids and D-amino acid oxidase were purchased from Sigma (St. Louis, MO, U.S.A.). The mobile phases generally contained various concentrations of acetonitrile in buffer (pH 7.0), containing 5.0 m*M* of L-proline, 2.5 m*M* of copper sulfate and 2.0 g/l of ammonium acetate.

Instrumentation

The HPLC system consisted of two Altex (Berkeley, CA, U.S.A.) 110A pumps, an Altex 420 gradient microprocessor and a Rheodyne (Cotati, CA, U.S.A.) 7105 injection valve. The analytical column, 15 × 0.42 cm I.D., was packed with Nucleosil 5 C_{18} (Duren, F.R.G.) by the downward slurry technique. The dansyl amino acids were detected with a Fluoro-tec filter fluorometer (American Research Products, Kensington, MD, U.S.A.). The amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.).

Reaction with D-amino acid oxidase

To assay the activity of the enzyme, a stock solution containing 2 mg D,L-alanine in 1 ml of 0.02 *M* potassium pyrophosphate (pH 8.3) was prepared. To 1 ml of this solution was added 200 μl of a solution containing 20 mg of D-amino acid oxidase and 5 mg of catalase in 1 ml of same buffer. The mixture was incubated at 37°C. The reaction of D-alanine with the enzyme was followed as a function of time by withdrawing 100-μl aliquots of the substrate every 15 min, synthesizing the dansyl derivative and assaying by HPLC. Dansylation was effected by adding 100 μl of the substrate to 400 μl of lithium carbonate followed by 200 μl of dansyl chloride (1.0 mg/ml in acetonitrile). At 100°C, dansylation was complete in 20 min. The same procedure was used to treat an aliquot of the complex mixture to be assayed.

Reaction of amino acid with racemase

A 10-ml aliquot of a *S. faecalis* culture was centrifuged at 3000 *g* for 15 min, and the cells were harvested, after three washings with 2-ml portions of isotonic saline. To the cells were added 2 ml of phosphate buffer (pH 8.1), 200 μl of pyridoxal phosphate (1 mg/ml) and 1 ml of D,L-alanine (2 mg/ml). The cells were then ruptured by ultrasonication in the presence of glass beads and the mixture was incubated at 37°C. To monitor the course of the reaction, 100-μl portions were removed every 30 min, dansyl derivatives of the amino acids were prepared, and aliquots were assayed by HPLC.

RESULTS

When a mixture of D,L-alanine was incubated with D-amino acid oxidase, the peak corresponding to the D-isomer gradually decreased in size as the D-alanine was oxidatively deaminated (Figs. 1 and 2). This was taken as confirmation of the separation of the D- and L-isomers and of the identification of the D-isomer by its

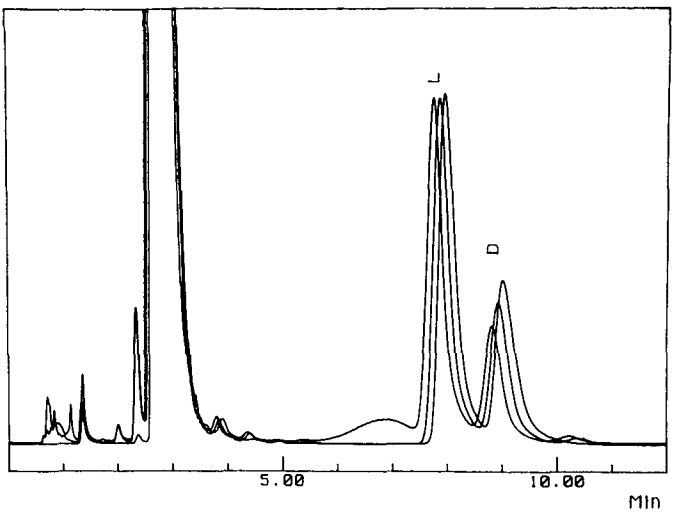

Fig. 1. Chromatogram of D,L-alanine. The size of the D-alanine peak decreased gradually as D-alanine was oxidatively deaminated at 0, 1 and 2 h. Mobile phase: 15% acetonitrile in a buffer (pH 7.0) containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 2.0 g ammonium acetate. Flow-rate, 2.0 ml/min.

retention time. With the enzyme used, there was no detectable reaction with the L-isomer. Incubation of a synthetic mixture, containing many D- and L-amino acids, with D-amino oxidase, followed by chiral HPLC, similarly showed marked diminution in size of the D-isomers (Fig. 3).

When a racemic mixture of alanine was incubated with the cell extract of *S. faecalis*, the L-isomer was racemized to the D-isomer until a constant proportion was

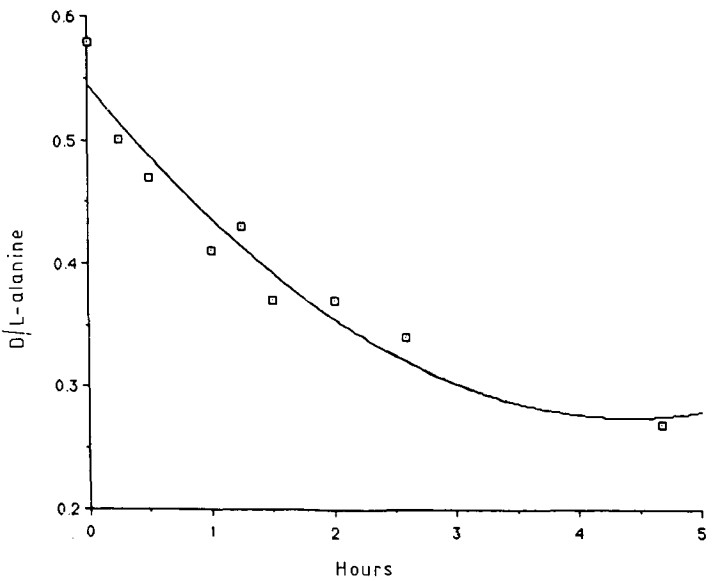

Fig. 2. Reaction kinetics of D-alanine with D-amino acid oxidase.

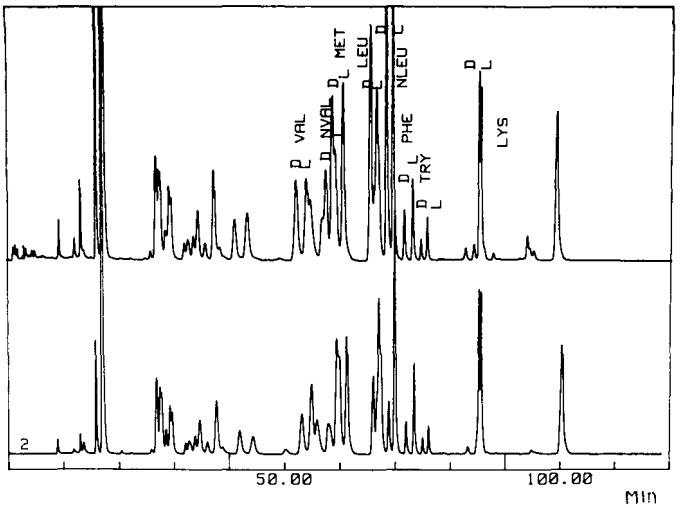

Fig. 3. Chromatogram of an amino acid mixture before (upper tracing) and after incubation with D-amino acid oxidase. Oxidative deamination of the D-isomers resulted in reduction in size of peaks corresponding to the D-amino acids. Mobile phase: 5.0 m*M* L-histidine methyl ester, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 2.0 g of ammonium acetate, pH 5.5. A stepwise gradient was formed by blending the buffer with a 45% acetonitrile solution of the same buffer.

reached at equilibrium (Fig. 4). The same racemase reacted with either D- or L-alanine, giving a peak corresponding to the opposite isomer. After extended incubation, the same proportion of D- and L-isomers was obtained, regardless of whether the starting material was the D- or L-form (Fig. 5). There was no reaction of this racemase with serine, valine, leucine and methionine. Other microbacterial systems contain racemases similarly specific for other amino acids.

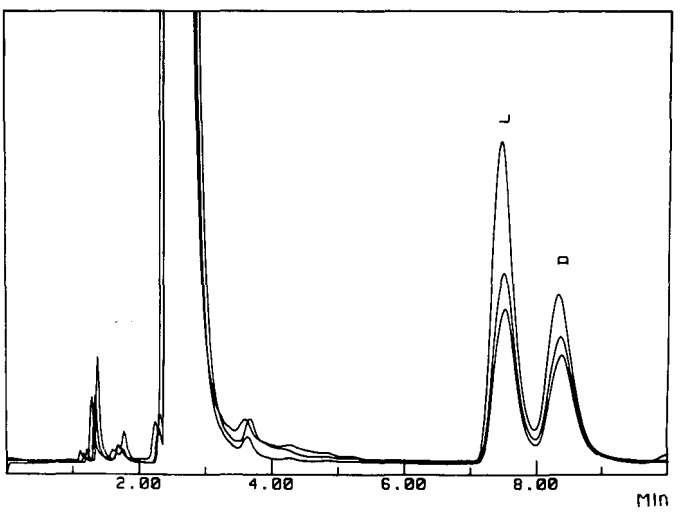

Fig. 4. Chromatogram of a racemic mixture of D,L-alanine incubated with cell extracts of *S. facecalis*. Constant D-to-L-alanine ratio was obtained at equilibrium (lower tracing). Conditions as in Fig. 1.

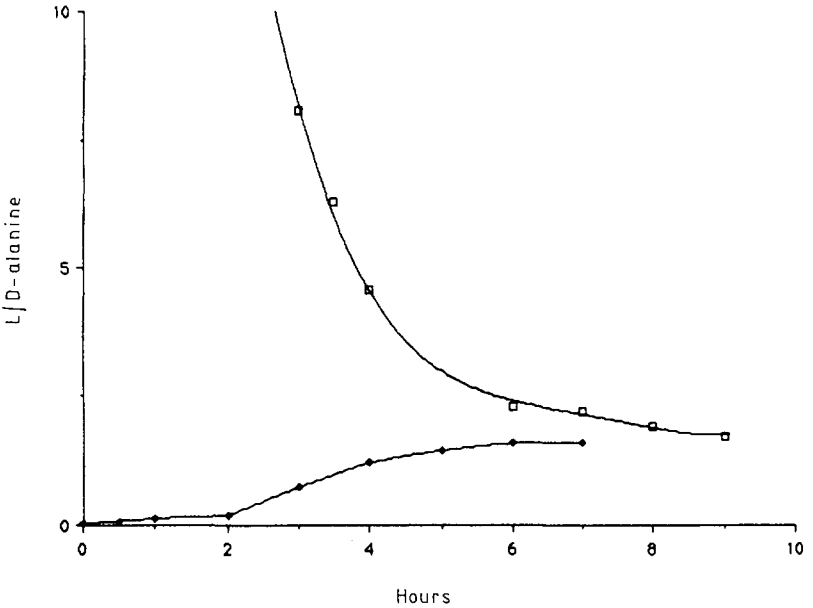

Fig. 5. Reaction kinetics of D- (◆) and L-alanine (□) with racemase.

DISCUSSION

D-Amino acid oxidase, used in the procedure described here, helped confirm the identification of D-amino acid isomers in chromatographic assays of complex mixtures. This enzyme is highly reactive with D-isomers and reacts with many different amino acids. For more specific identification of individual amino acids in a mixture, the racemase family of enzymes offers much greater specificity for individual amino acid pairs.

Different bacteria have racemases with different specificities toward amino acids. Chiral HPLC could obviously be used to characterize the racemases in cultures of individual bacterial species by incubating the bacteria with a mixture of L- or D-amino acids and then assaying the reaction mixture for the other isomers. We are not aware of anyone attempting to use the specificity of the racemases as a means of identifying or characterizing the bacteria.

We have also considered the use of D-amino acid oxidase in a post-column reactor for specific detection of the D-amino acids in a mixture. Highly sensitive enzymatic methods, based on specific oxidases, are widely used in clinical chemistry for assaying such metabolites as glucose, glycerol and cholesterol. For use in HPLC, reactors packed with an immobilized enzyme that is relatively resistant to denaturation by the mobile phase are generally preferred to adding soluble enzyme, if only for economic reasons. We have used post-column reactors containing semi-specific immobilized enzymes for highly specific and sensitive detection in HPLC of bile acids and steroid hormones[8]. For use with the system of amino acid HPLC described here, a post-column enzymatic detection method may require a method for reducing the

concentration of potentially toxic cupric ions in the HPLC effluent. This would be unnecessary if the HPLC separation of isomers were performed with a chiral stationary phase rather than with the copper complex method used here.

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CHROMSYMP. 1548

WALL-COATED ADSORBENT OPEN-TUBULAR FUSED-SILICA COLUMNS FOR THE SEPARATION OF COMPLEX PYROLYSIS PRODUCTS

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SUMMARY

Fused-silica capillary columns coated with a layer of non-porous, homogeneous graphitized carbon black, modified with a variety of stationary phases, were prepared. A series of parameters were determined. The selectivity and efficiency of the columns were much improved. These columns were used in the analysis of complex pyrolysis products and the separation of isomers.

INTRODUCTION

Gas–solid chromatography (GSC) has advantages over gas–liquid chromatography (GLC) owing to the high separation factors obtained. However, a drawback is that the elution temperature is usually much higher than that in GLC. For this reason, the use of GSC has been limited to the analysis of permanent gases or low-boiling hydrocarbons. The introduction of graphitized carbon black (GCB), with a suitable amount of liquid stationary phase added, has made liquid-modified GSC competitive with GLC, even with regard to elution temperature[1].

Glass capillary columns coated with carbon black were introduced several years ago by Vidal Madjar *et al.*[2] and Goretti *et al.*[3]. Recently, Bruner *et al.*[4] showed that improved results are obtained by static coating of the inner surface of capillary columns with GCB impregnated with the liquid phase SP-1000. In this study, wall-coated adsorbent open-tubular (WCAOT) columns were prepared with coatings of Carbopack F (B) and Carbonsieve TDX, modified with the stationary phases DC-550, OV-17 and PEG 20M. The selectivity due to the mechanism of gas–liquid–solid chromatography (GLSC) was much improved and the efficiency in terms of HETP was enhanced. By coupling of the high separation factors due to GLSC with the high efficiency of GLC capillary columns, these columns can be used for separating complex pyrolysis products and separating isomers.

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EXPERIMENTAL

Measurements were performed with a Sigma 1B gas chromatograph, coupled with a Sigma 10 microprocessing system (Perkin-Elmer, Norwalk, CT, U.S.A.) and a GC-5A gas chromatograph (Shimadzu, Kyoto, Japan), using hydrogen as the carrier gas. A JMS-D300 gas chromatograph–mass spectrometer (JEOL, Tokyo, Japan) was used for qualitative analysis. Carbopack F and B were obtained from Supelco (Bellefonte, PA, U.S.A.). STH-01 graphitized carbon black (Jilin Carbon Black Factory, Jilin, China) and carbonsieve TDX (Tianjing No. 2 Reagents Plant, China) were used. Fused-silica tubing was obtained from the Hebei Light-Fibre Factory (Hebei, China). The stationary phases DC-550, OV-17, PEG 20M and picric acid were obtained from the Beijing Chemical Agent Shop (Beijing, China).

The method used for the preparation of the column, a modification of that described by Xu and Vermeulen[5], consists in coating by the static method, keeping the column at a temperature higher than the boiling point of the solvent.

The two kinds of carbon black were first ground in a medium of 95% ethanol and then fractionated by flotation with ethanol to obtain fines of < 1 μm. A mixture of volatile pentane and methyl chloride (1:2) was chosen as the solvent. A slurry of 300 mg of Carbopack F or B and 80 ml of mixed solvent, with suitable amounts of stationary phase added, was treated by ultrasound for 40 min in a US-150 Ultrasonic device. In this way, the size of carbon black particles was further reduced to about 0.2 μm (see Fig. 1).

A 25 m × 0.25 mm I.D. fused-silica capillary column was filled with a slurry of GCB by nitrogen pressure using a Micro-column Treating Stand (Shimadzu, Kyoto, Japan). After the capillary column had been completely filled, one end was carefully sealed with stearin and the other end was connected via shrinkable PTFE tubing to a 25 m × 0.25 mm I.D. glass capillary column, which served as a damping column. By immersing the columns in a water-bath (75°C), the coating process was started with the evolution of fine bubbles. After 5–6 h, when the solvent had completely evaporated, nitrogen was passed through the column for 1 h, then the column was ready for conditioning overnight at the maximum operating temperature of the stationary phase.

The samples to be analysed were the products of gas-oil pyrolysis, *viz.*, light hydrocarbons (C_1–C_6), pyrolysis gasoline and fuel oil, and were tested separately.

RESULTS AND DISCUSSION

In Fig. 2, a typical Van Deemter plot is shown for two columns of the same size (20 m × 0.25 mm I.D.) and the same stationary phase, but one was coated with Carbopack F (GLSC) modified with DC-550 and the other was a DC-550 (GLC) column. Comparative data are presented in Table I. A third column was prepared by coating Carbopack B with PEG 20M. This showed intermediate efficiency between the GLC and Carbopack F columns.

In Table I, the value of the separation number (TZ) is higher for the GLSC column (31.1 or 32.0) than for the GLC column (22.1). This shows that liquid-modified adsorption chromatography offers higher separation factors. The isosteric heat of adsorption (Q_{st}) was measured, and was 14.5 or 15.4 kcal/mol for the GLSC

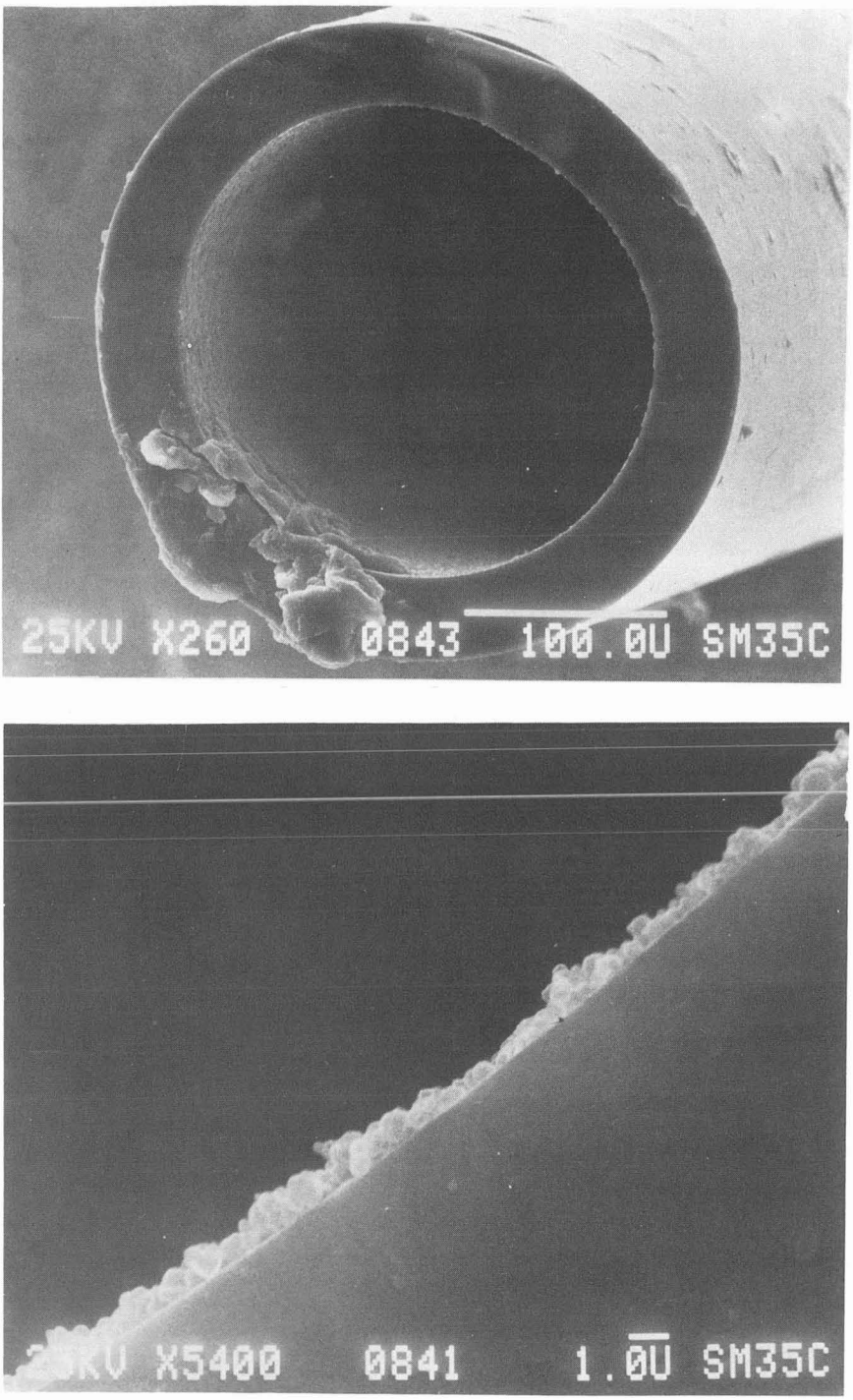

Fig. 1. Micrographs of the internal wall of the fused-silica capillary coated with Carbopack F and DC-550.

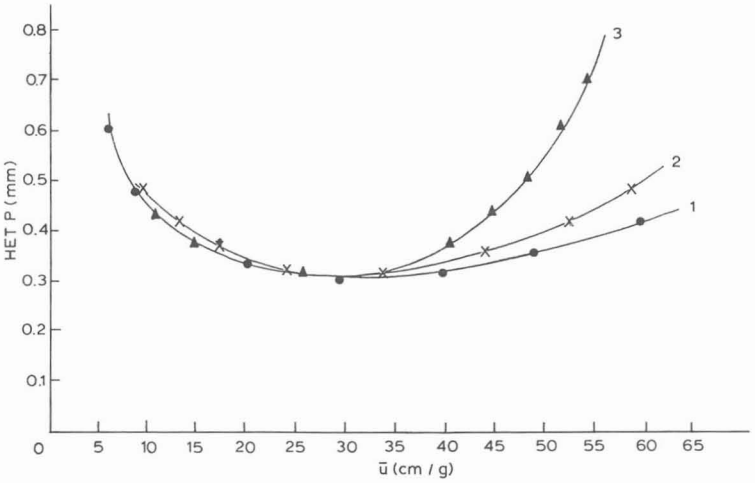

Fig. 2. Typical example of Van Deemter plot for Carbopack F (B), modified with Carbowax PEG 20M and DC-550. Sample: n-C_{12} at 135°C (k' = 6.5). Carrier gas: hydrogen. Lines 1–3 correspond to columns 1–3 in Table I.

column and 9.8 kcal/mol for the GLC column. The column with the higher Q_{st} was operating with a GLSC mechanism. This is further confirmed by the value obtained from bleeding of the stationary phase. For the GLSC column, the bleeding was lower by a factor of 3.4, which means that the GLSC column has good thermal stability. These columns have been used over a period of 6 months and no significant changes in retention time and selectivity have been observed.

Fig. 3 shows the separation of pyrolysis gasoline (b.p. <180°C cut), performed on the WCAOT column with Carbopack F modified with 10% DC-550. Pyrolysis gasoline is a complex mixture containing large amounts of paraffins, olefins, diolefins and aromatic hydrocarbons. Qualitative analysis was performed by gas chromatography–mass spectrometry and the use of Kováts retention indices. More than 93 components were identified. Compared with conventional GLC capillary columns with non-polar squalane, the analysis time with the GLSC column is reduced by one third, while the same resolution is achieved. Alkylbenzenes in pyrolysis gasoline are listed in

TABLE I

COMPARISON OF CHROMATOGRAPHIC PARAMETERS FOR GLSC AND GLC COLUMNS

Columns: 1 = fused-silica capillary (20 m × 0.25 mm I.D.) coated with Carbopack F + 10% DC-550 (GLSC 1); 2 = fused-silica capillary (20 m × 0.25 mm I.D.) coated with Carbopack B + 15% PEG 20M (GLSC 2); 3 = fused-silica capillary (20 m × 0.25 mm I.D.) coated with 10% DC-550, film thickness 0.5 μm (GLC).

Column No.	H_{min} *(mm)*	$\bar{u}$ *(cm/s)*	*TZ* (k' = 6)	*Coating efficiency (%)*	$k'_{(n\text{-}C_{16})}$ *(105°C)*	$Q_{st\ (n\text{-}C_{12})}$ *(kcal/mol)*	*Bleeding at 240°C (% full-scale)*
1	0.29	35	31.1	76	5.2	14.5	6
2	0.32	34	32.0	70	5.0	15.4	5
3	0.29	34	22.1	76	8.5	9.8	18

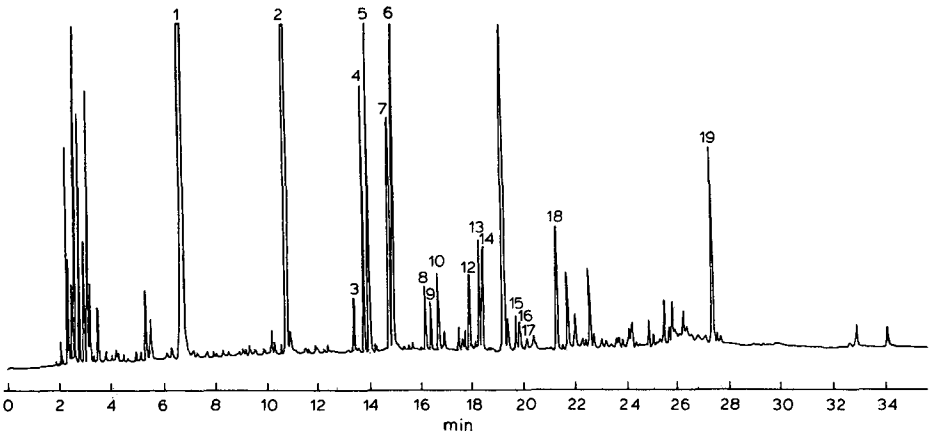

Fig. 3. Separation of the pyrolysis gasoline (b.p. < 180°C cut). Fused-silica capillary column, 20 m × 0.25 mm I.D., Carbopack F + DC-550. Temperature programme, 40°C for 5 min, raised at 4°C/min to 180°C. For peak identifications, see Table II.

Table II. By increasing the polarity of the stationary phase and using Carbowax PEG 20M, paraffins, naphthenes and olefins were eluted before benzene, methylbenzene and dimethylbenzene and dimethylbenzene isomers were easily separated. The column is suitable for the determination of hydrocarbon types.

Fig. 4 shows a chromatogram of pyrolysis fuel oil (b.p. 180–300°C). In order to determine the amounts of individual alkylnaphthalenes present, a conventional method of analysis was established. C_{10}–C_{12} alkylnaphthalenes were isolated from the fuel oil by liquid chromatography and separated on a WCAOT column with Carbopack B modified with OV-17. Naphthalene, two methylnaphthalenes, two ethylnaphthalenes and eight dimethylnaphthalenes were identified. The separation of alkylnaphthalene mixtures has been reported by numerous investigators[6,7]; it seems to be difficult to separate all the compounds (see Table III) because they have similar structures. In this work, owing to the GSC mechanism, the column used is superior in plate number

TABLE II

DETERMINATION OF ALKYLBENZENES IN PYROLYSIS GASOLINE

Peak No.	*Compound*	*Wt. – %*	*Peak No.*	*Compound*	*Wt. – %*
1	Benzene	19.2	11	1,3,5-Trimethylbenzene	0.4
2	Toluene	18.5	12	1-Methyl-2-ethylbenzene	0.5
3	Ethylbenzene	2.5	13	1,2,4-Trimethylbenzene	0.2
4	*p*-Xylene	1.4	14	α-Methylstyrene	0.3
5	*m*-Xylene	3.7	15	1,3-Methylisopropylbenzene	0.5
6	Styrene	7.8	16	*n*-Butylbenzene	0.3
7	*o*-Xylene	1.7	17	Indane	0.5
8	Isopropylbenzene	0.5	18	Indene	3.1
9	*n*-Propylbenzene	0.2	19	Naphthalene	2.5
10	1-Methyl-3-ethylbenzene	1.3			

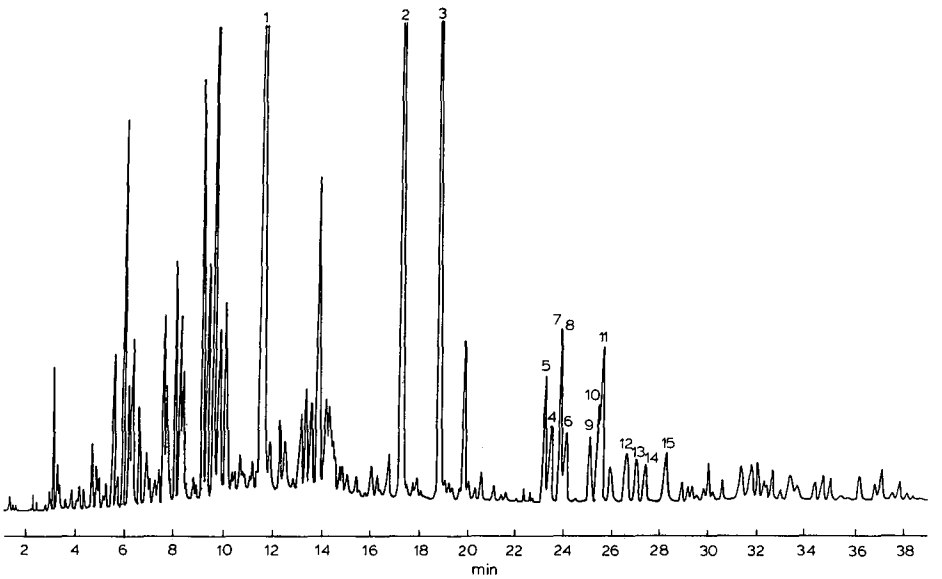

Fig. 4. Separation of pyrolysis fuel oil (b.p. 180–300°C cut). Fused-silica capillary column, 20 m × 0.25 mm I.D., Carbopack B + OV-17. Temperature programme, 95°C for 10 min, raised at 4°C/min to 200°C. For peak identifications, see Table III.

and separation factors to those used previously[6,7]; 2,6- and 2,7-dimethylnaphthalene (DMN) are hardly separated, 1,6- and 1,3-DMN are partially separated and 2,3-, 1,4-, 1,5- and 1,2-DMN are completely separated (Fig. 5).

Fig. 6 shows the separation of pyrolysis gas on a fused-silica capillary column coated with carbon sieve (TDX). The elution order follows the carbon number. C_1–C_5 hydrocarbons are well separated, except the butene isomers. It is difficult to separate butene isomers by using only carbon black as the stationary phase.

TABLE III

ALKYLNAPHTHALENES ISOLATED FROM PYROLYSIS FUEL OIL (b.p. 180–300°C)

Peak No.	*Compound*[a]	*RRT*[b]	*b.p. (°C)*	*Peak No.*	*Compound*[a]	*RRT*[b]	*b.p. (°C)*
1	Naphthalene	1.00	217.96	9	1,7-DMN	2.87	262.90
2	2-MN	1.65	241.14	10	1,3-DMN	2.95	265.50
3	1-MN	1.87	244.18	11	1,6-DMN	2.99	265.5
4	Diphenyl	2.54	255.00	12	2,3-DMN	3.20	268.0
5	2-EN	2.50	257.90	13	1,4-DMN	3.30	268.5
6	1-EN	2.65	258.67	14	1,5-DMN	3.37	270.1
7	2,6-DMN	2.61	262.00	15	1,2-DMN	3.57	271.1
8	2,7-DMN	2.62	262.00	16	Acenaphthane	4.20	277.2

[a] MN = methylnaphthalene; EN = ethylnaphthalene; DMN = dimethylnaphthalene.

[b] RRT = relative retention time (naphthalene = 1.00).The retention time of naphthalene was 11.7 min.

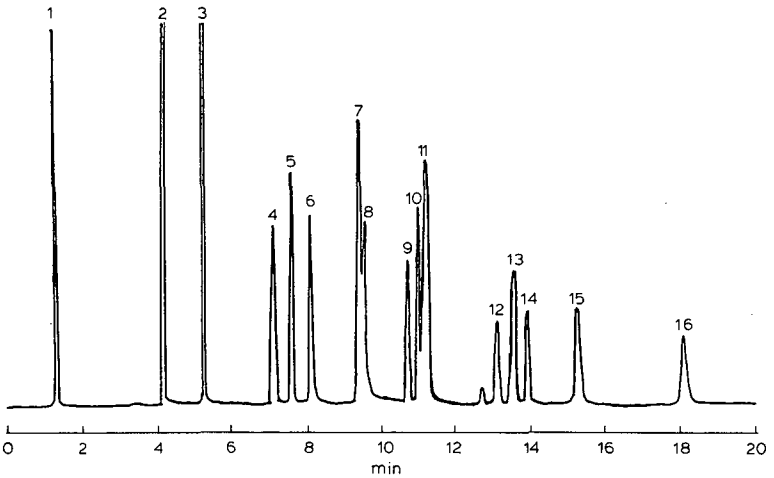

Fig. 5. Separation of C_{10}–C_{12} alkylnaphthalenes. Fused-silica capillary column, 20 m × 0.25 mm I.D., Carbopack B + DC-550. Temperature, 200°C. For peak identifications, see Table III.

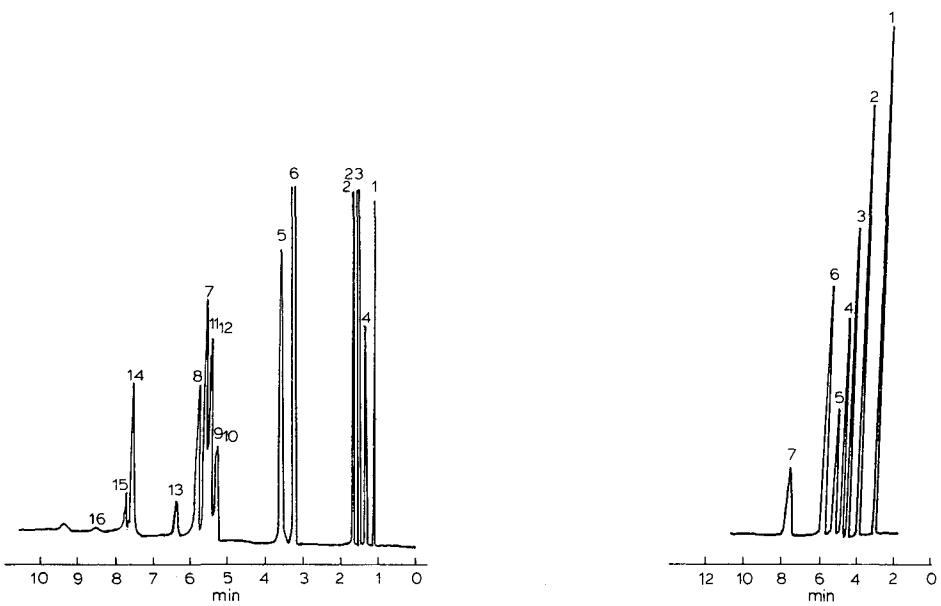

Fig. 6. Separation of C_1–C_5 hydrocarbons. Fused-silica capillary column, 10 m × 0.25 mm I.D., carbon molecular sieve (TDX). Temperature programme, 40°C for 2 min, raised at 25°C/min to 350°C. Peaks: 1 = methane; 2 = ethane; 3 = ethylene; 4 = acetylene; 5 = propane; 6 = propylene; 7 = isobutane; 8 = *n*-butane; 9 = isobutene; 10 = 1-butene; 11 = *trans*-2-butene; 12 = *cis*-2-butene; 13 = 1,3-butadiene; 14 = isopentane; 15 = *n*-pentane; 16 = 1-pentene.

Fig. 7. Separation of C_4 hydrocarbons. Fused-silica capillary column, 8 m × 0.25 mm I.D., STH-01 + 0.2% picric acid. Temperature, 30°C. Peaks: 1 = isobutene; 2 = *n*-butene; 3 = *n*-butane; 4 = isobutene; 5 = *cis*-2-butene; 6 = *trans*-2-butene; 7 = 1,3-butadiene.

Using STH-01 modified with 0.2% picric acid, seven isomers were completely separated at room temperature (Fig. 7).

These examples have shown that, by using a coating of a non-porous, non-specific adsorbent, such as GCB, with a polar stationary phase, the analytical potential of GSC is preserved to a great extent when non-polar hydrocarbons are eluted. This is due to the fact that if the molecules to be eluted are poorly soluble in the liquid stationary phase, the effect of the liquid modifier is to deactivate the surface and to reduce the retention time. Further, the interactions of the analyte molecules with the adsorbent–modifier system are such that a higher selectivity can be achieved than with the pure adsorbent.

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CHROMSYMP. 1560

DISPERSION AND SELECTIVITY INDICES IN GAS CHROMATOGRAPHY

IV[a]. CHLORINATED AROMATIC COMPOUNDS

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SUMMARY

The dispersion (I_M) and selectivity (I^*) indices of several series of chlorinated aromatic compounds, including chlorinated benzenes, anisoles (methoxybenzenes), veratroles (1,2-dimethoxybenzenes), phenyl acetates, benzaldehydes, 2-hydroxybenzaldehydes (salicylaldehydes), 4-hydroxybenzaldehydes and phenols, were determined on low-polarity (SE-30) and polar capillary columns. The effect of the individual substituent groups and of multiple substituents and their positions on the aromatic ring are discussed in terms of the relevant polar interactions. The results are compared with those previously reported for aliphatic and aromatic esters.

INTRODUCTION

The division of the Kováts retention index (I) of a solute in gas chromatography into two characteristic components has recently been reported[1]. The dispersion index (I_M), indicative of the contribution to retention of the non-polar forces, is related to the molecular weight of a particular solute. The second contribution, the selectivity index (I^*), indicates the polar interactions present, moderated by operative steric effects. The selectivity index was developed by considering the behaviour of a wide range of aliphatic and some aromatic carbonyl compounds. These included normal, branched and unsaturated aliphatic esters with variations of the structural parameters in both the acyl and alkyl chains, simple ketones (2-alkanones), diketones and homologous pyruvate esters, containing both a carboxy and a carbonyl functional group[2]. The aromatic esters were the alkyl, 2-chloroethyl and alkenyl normal and branched-chain benzoate esters and their chlorinated derivatives[3].

This work extends the treatment to a wide range of aromatic compounds, including all of the isomeric chlorobenzenes[4], anisole (methoxybenzene)[5], veratrole (1,2-dimethoxybenzene)[6], phenyl acetate[7], 2-hydroxybenzaldehyde (salicylalde-

[a] For Part III, see ref. 3.

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hyde)[8], 4-hydroxybenzaldehyde[9], phenol[10] and their chlorinated derivatives on non-polar (SE-30) and polar capillary columns. The results of this study are compared and discussed in terms of the operative polar interactions with electron-donating and accepting groups about the aromatic nucleus. The retention data used in this work were taken from earlier retention studies of the compounds of interest and some minor correlative data[4–10].

EXPERIMENTAL

The retention indices were determined with Varian Aerograph 2400 and Perkin-Elmer Sigma 3 instruments under the previously reported conditions[4–10]. The columns used were a vitreous silica capillary column (25 m × 0.32 mm I.D.) coated with SE-30 (SGE, North Melbourne, Australia). The polar column used for the chlorobenzenes was a glass capillary column (22 m × 0.3 mm I.D.), coated with Carbowax 20M (SGE) and for the phenols a vitreous silica column (25 m × 0.35 mm I.D.) coated with FFAP (SGE) was used. The polar capillary column used for the other compounds was of fused silica (25 m × 0.32 mm I.D.) coated with OV-351 (Orion Analytica, Espoo, Finland).

The column temperature was 160°C in all instances and the retention indices were calculated by computerized procedures, as reported previously[4,5].

RESULTS AND DISCUSSION

The dispersion (I_M) and selectivity (I^*) indices for the chlorobenzenes on the SE-30 column are given in Table I. It is evident that the I_M values increase with

TABLE I

DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES OF CHLORINATED BENZENES ON SE-30 AND CARBOWAX 20M AT 160°C

Isomer	*SE-30*			*Carbowax 20 M*	
	I	I_M	I^*	I	I^*
Monochloro-	832	788.1	43.9	1257	468.9
1,3-Dichloro-	964	1033.6	−69.6	1415	381.4
1,4-Dichloro-	970	1033.6	−63.6	1438	404.4
1,2-Dichloro-	1005	1033.6	−28.6	1445	413.4
1,3,5-Trichloro-	1131	1279.2	−148.2	1515	235.8
1,2,4-Trichloro-	1177	1279.2	−102.2	1630	350.8
1,2,3-Trichloro-	1211	1279.2	−68.2	1705	425.8
1,2,3,5-Tetrachloro-	1326	1527.7	−201.7	1754	226.3
1,2,4,5-Tetrachloro-	1326	1527.7	−201.7	1764	236.3
1,2,3,4-Tetrachloro-	1366	1527.7	−161.7	1871	343.3
Pentachloro-	1496	1770.3	−274.3	1956	185.7
Hexachloro-	1656	2015.9	−359.9	2141	108.1

addition of chlorine and the corresponding increase in molecular weight. The selectivity indices decrease substantially with added chlorine substituents. The value for benzene is not shown, because it is difficult to measure accurately the retention of this low-boiling solute at 160°C. However, the retention index is well known, having a value of *ca.* 660 (SE-30) and a selectivity index of *ca.* 120.

The I^* value of monochlorobenzene is reduced (43.9) but remains positive. The addition of further chlorine substituents produces progressive decreases in I^* due, as indicated previously[1,4], to the effect of the screened electrons of the halogen atoms. As has been discussed previously, the greatest retention occurs with the isomers in which the substituents are closest together and decreases as the position around the ring allows greater separation[4]. Thus, the I and I^* values for the homologues follow the sequence 1,2- < 1,4- < 1,3-.

TABLE II

DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES OF ANISOLE AND VERATROLE AND THEIR CHLORINATED DERIVATIVES ON SE-30 AND OV-351 AT 160°C

Compound	I_M	I^*	
		SE-30	*OV-351*
Anisole	756.6	123.4	616.4
2-Chloro-	1002.1	96.9	687.9
3-Chloro-	1002.1	82.9	625.9
4-Chloro-	1002.1	91.9	653.9
2,3-Dichloro-	1247.7	61.3	729.3
2,4-Dichloro-	1247.7	24.3	657.3
2,5-Dichloro-	1247.7	16.3	644.3
2,6-Dichloro-	1247.7	−49.7	484.3
3,4-Dichloro-	1247.7	37.3	651.3
3,5-Dichloro-	1247.7	−4.7	552.3
2,3,4-Trichloro-	1493.3	−5.3	710.7
2,3,5-Trichloro-	1493.3	−56.3	577.7
2,3,6-Trichloro-	1493.3	−122.3	449.7
2,4,5-Trichloro-	1493.3	−70.3	586.7
2,4,6-Trichloro-	1493.3	−160.3	343.7
3,4,5-Trichloro-	1493.3	−39.3	586.7
2,3,4,5-Tetrachloro-	1738.8	−105.8	601.2
2,3,4,6-Tetrachloro-	1738.8	−218.8	332.2
2,3,5,6-Tetrachloro-	1738.8	−220.8	332.2
2,3,4,5,6-Pentachloro-	1984.4	−285.4	294.6
Veratrole	970.6	143.4	732.4
3-Chloro-	1216.2	17.3	646.8
4-Chloro-	1216.2	56.3	742.8
3,4-Dichloro-	1461.8	−10.8	665.2
3,5-Dichloro-	1461.8	−49.8	563.2
3,6-Dichloro-	1461.8	−18.8	424.2
4,5-Dichloro-	1461.8	−21.2	741.2
3,4,5-Trichloro-	1703.3	−94.3	604.7
3,4,6-Trichloro-	1703.3	−204.3	352.7
3,4,5,6-Tetrachloro-	1952.9	−262.9	348.1

TABLE III

EFFECT OF ADJACENT GROUPS OF THE SAME AND DIFFERENT TYPES ON SELECTIVITY IN CHLORINATED AROMATIC DERIVATIVES

Number of pendant groups	*I**	*Same functional group*		*Different functional group*	
		Chlorobenzenes	*Veratroles*	*Phenyl acetates*	*Anisoles*
2	Max.	1,2-Dichloro-[a]	–	4-Chloro-[a]	2-Chloro-[a]
	Min.	1,3-Dichloro-[a]	–	2-Chloro-[a]	3-Chloro-[a]
3	Max.	1,2,3-Trichloro-[a]	4-Chloro-[a]	3,4-Dichloro-[a]	2,3-Dichloro-[a]
	Min.	1,3,5-Trichloro-[a]	3-Chloro-[a]	2,6-Dichloro-[b] 3,5-Dichloro-[c]	2,6-Dichloro-[a]
4	Max.	1,2,3,4-Tetrachloro-[a]	3,4-Dichloro-[b] 4,5-Dichloro-[c]	3,4,5-Trichloro-[b] 2,3,4-Trichloro-[c]	2,3,4-Trichloro-[a]
	Min.	1,2,3,5-Tetrachloro-[c] 1,2,4,5-Tetrachloro-[b]	3,5-Dichloro-[b] 3,6-Dichloro-[c]	2,4,6-Trichloro[a]	2,4,6-Trichloro-[a]

[a] Both phases.
[b] Non-polar phase.
[c] Polar phase.

On the polar stationary phase, the retention indices are higher for all of the compounds, and accordingly the I^* values are also higher. It is apparent that on both phases I^* decreases with increasing extent of chlorination, the increased screening effect being responsible. This effect has been previously observed in terms of retention increments.

The dispersion and selectivity indices for anisole and veratrole and each of their chlorinated products are shown in Table II. The chlorinated anisoles and veratroles generally follow the elution pattern of the chlorobenzenes. This pattern is very similar on both the low-polarity and polar phases, with only minor variations, which when they occur, are small.

The anisole compounds on SE-30 show considerably higher values of I^* than the chlorinated benzenes. This effect is expected from the structures, the chlorobenzenes having substantial polar groups attached to the aromatic nucleus of donor character and also possessing a π-bonding system. The anisoles with the pendant methoxy group have a localized donor site, and the acceptor and shielding effects are reduced. On the acceptor phase, the values of I^* for both series are increased substantially, but those for the anisoles remain highest. On both phases, the species with maximum and minimum retentions and I^* are as shown in Table III.

It is apparent that the lowest values of I^* occur for the isomers with two similar groups, shielding another functional group. The situation is the opposite of that in which the pendant groups are the same and enhancement of retention occurs, as shown in Table III for the chlorobenzenes.

With the polar phase, all of the I^* values are increased as expected; with the lower degrees of substitution the same trend occurs as on SE-30, but as the degree of substitution increases, the values of I^* for the anisoles become greater than those for the veratroles. On the electron-withdrawing phase, the effect for the compounds with more acceptor groups becomes predominant.

The relative effect of *o*-chloro and *p*-methoxy substitution on isomeric anisoles was reported by Korhonen[11]. He observed that the incremental effects of an additional chlorine atom in isomeric veratroles were generally lower than those for anisoles. The methoxy group had a greater effect than a chlorine atom, each substituted in the *ortho*-position without an adjacent chlorine atom. Lower retention enhancements were observed with the methoxy group adjacent to the chlorine atom than with the corresponding chlorine substitution. The I^* values shown in Table II further illustrate these observations.

The selectivity indices for the chlorinated phenyl acetates are shown in Table IV. They decrease substantially with added chlorine substituents. The values of I^* on the non-polar stationary phase are similar to those of the chlorobenzene isomers, whereas on OV-351 the values are higher. The elution trends of the various isomers closely parallel those of the chlorobenzenes. This behaviour can be reasonably explained, as discussed in earlier work[4].

The retention (I), dispersion (I_M) and selectivity (I^*) indices of phenol and of the various chlorinated phenols, determined on SE-30 and FFAP at 160°C, are shown in Table V.

On SE-30, monochlorination causes a small increase in I. However, the dispersion indices also increase, owing to their relationship with molecular weight. The I^* value of 2-chlorophenol is greatly reduced compared with phenol. The strongly

TABLE IV

DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES OF CHLORINATED PHENYL ACETATES ON SE-30 AND OV-351 AT 160°C

Compound	*I (SE-30)*	I_M	*I* (SE-30)*	*I (OV-351)*	*I* (OV-351)*
Phenyl acetate	1008	956.3	51.7	1633	676.7
2-Chloro-	1173	1201.8	−28.8	1829	627.2
3-Chloro-	1198	1201.8	−3.8	1848	646.2
4-Chloro-	1203	1201.8	1.2	1870	668.2
2,6-Dichloro-	1296	1447.4	−151.4	1971	523.6
2,4- + 2,5-Dichloro-	1317	1447.4	−130.4	1986	538.6
3,5-Dichloro-	1334	1447.4	−113.4	1964	516.6
2,3-Dichloro-	1350	1447.4	−97.4	2052	604.6
3,4-Dichloro-	1377	1447.4	−70.4	2072	624.6
2,4,6-Trichloro-	1420	1693.0	−273.0	2035	342.0
2,3,6-Trichloro-	1463	1693.0	−230.0	2139	446.0
2,3,5-Trichloro-	1476	1693.0	−217.0	2117	424.0
2,4,5-Trichloro-	1481	1693.0	−212.0	2133	440.0
2,3,4-Trichloro-	1525	1693.0	−168.0	2233	540.0
3,4,5-Trichloro-	1543	1693.0	−150.0	2215	522.0
2,3,5,6-Tetrachloro-	1612	1938.5	−326.5	2225	286.5
2,3,4,6-Tetrachloro-	1617	1938.5	−321.5	2236	297.5
2,3,4,5-Tetrachloro-	1679	1938.5	−259.5	2233	394.5
2,3,4,5,6-Pentachloro-	1800	2184.1	−370.6	2401	216.9

polar hydroxy group adjacent to the halogen with screened electrons tends to reduce greatly the polar effects of the hydroxy group. However, chlorination in the 3- and 4-positions has little effect on the hydroxy group, as I^* is only slightly reduced. For these positions, the effect of the hydroxy group predominates.

Of the dichlorophenols, all four isomers with one chlorine atom adjacent to the hydroxy group exhibit I^* values that are greatly reduced, largely owing to the increased value of I_M. The 3,4- and 3,5-dichlorophenol isomers show considerably enhanced retentions and similarly I^* values. The I^* values of the 3,4- and 3,5-isomers are slightly reduced compared with those of the 3- and 4-chloro isomers, owing to the effect of the added chlorine substituent. The chlorine atom continues to have a dramatic effect when adjacent to the hydroxy group.

For the trichlorophenols, the additional chlorine atom in the isomers with a halogen adjacent to the hydroxy group has a considerable effect, and the I^* values are considerably reduced. 3,4,5-Trichlorophenol, with the halogens isolated from the hydroxy group, has considerably greater retention, and the I^* value is only slightly lower than those for 3,4- and 3,5-dichlorophenol. The reduction is again due to the small additional effect of the chlorine atom in this particular isomer.

For the tetra- and pentachlorophenols, the predominant effect of the additional halogen atoms is that due to the position of the groups, although one chlorine atom is adjacent to the hydroxy group.

TABLE V

DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES OF PHENOL AND CHLORINATED PHENOLS ON SE-30 AND FFAP AT 160°C

Compound	*I (SE-30)*	I_M	*I* (SE-30)*	*I (FFAP)*	*I* (FFAP)*
Phenol	929	656.6	272.4	2000	1343.4
2-Chloro-	991	902.1	88.9	1866	963.9
3-Chloro-	1157	902.1	254.9	2371	1468.9
4-Chloro-	1157	902.1	254.9	2371	1468.9
2,3-Dichloro-	1180	1147.7	32.3	2160	1012.3
2,4-Dichloro-	1170	1147.7	22.3	2151	1003.3
2,5-Dichloro-	1170	1147.7	22.3	2160	1012.3
2,6-Dichloro-	1200	1147.7	52.3	2097	1049.3
3,4-Dichloro-	1378	1147.7	230.3	2731	1583.3
3,5-Dichloro-	1375	1147.7	227.3	2675	1527.3
2,3,4-Trichloro-	1363	1393.3	−30.3	2453	1059.7
2,3,5-Trichloro-	1327	1393.3	−66.3	2403	1009.7
2,3,6-Trichloro-	1354	1393.3	−39.3	2368	974.7
2,4,5-Trichloro-	1356	1393.3	−37.3	2458	1064.7
2,4,6-Trichloro-	1346	1393.3	−47.3	2301	907.7
3,4,5-Trichloro-	1587	1393.3	193.7	3028	1634.7
2,3,4,5-Tetrachloro-	1536	1638.8	−102.8	2730	1091.2
2,3,4,6-Tetrachloro-	1538	1638.8	−100.8	2554	915.2
2,3,5,6-Tetrachloro-	1530	1638.8	−108.8	2553	914.2
2,3,4,5,6-Pentachloro-	1720	1884.4	−164.4	2821	936.6

On the polar phase, the retentions of all isomers are much greater than those on SE-30 and, similarly, all of the I^* values are increased. Overall, the effect of the halogen atoms tends to predominate more strongly for the most sensitive isomers. The 3- and 4-chloro isomers, the 3,4- and 3,5-dichlorophenols and the 3,4,5-trichlorophenols have greater I^* values than phenol. The values for the tetra- and pentachlorophenols are much less reduced than for the same isomers on SE-30. On the polar phase, the elution pattern follows more closely the pattern proposed, so that 3,4-dichlorophenol is eluted after 3,5-dichlorophenol and 2,3,4,5-tetrachlorophenol after the other tetrachlorophenols.

Table VI shows the retention (I), dispersion (I_M) and selectivity (I^*) indices for the 2- and 4-hydroxybenzaldehydes and their chlorinated derivatives, and those for the parent compounds are shown in Table VII. In Table VII it is possible to compare the various indices of the parent compounds with those of compounds having one and two functional groups. On the low-polarity phase, the retention indices increase slightly from phenol to benzaldehyde and then more so with the two functional groups, the retention of the 2-hydroxy compound being appreciably lower than that of the 4-hydroxy compound. The strongly polar hydroxy group adjacent to the alde-

TABLE VI

DISPERSION (I_M) AND SELECTIVITY (I^*) INDICES OF CHLORINATED HYDROXYBENZALDEHYDES ON SE-30 AND OV-351 AT 160°C

Compound	*I (SE-30)*	I_M	*I* (SE-30)*	*I (OV-351)*	*I* (OV-351)*
Phenol	964	656.6	307.4		
Benzaldehyde	978	742.2	235.8		
4-Hydroxybenzaldehyde	1320	856.3	463.7		
2-Chloro-	1524	1101.8	422.2		
3-Chloro-	1291	1101.8	189.2		
2,3-Dichloro-	1463	1347.4	115.6		
2,5-Dichloro-	1449	1347.4	101.6		
2,6-Dichloro-	1753	1347.4	405.6		
3,5-Dichloro-	1465	1347.4	117.6		
2,3,5-Trichloro-	1632	1593.0	39.0		
2,3,6-Trichloro-	1651	1593.0	58.0		
Tetrachloro-	1820	1838.5	−18.5		
2-Hydroxybenzaldehyde	1062	856.3	205.7	1742	885.7
3-Chloro-	1264	1101.8	162.2	2082	980.2
4-Chloro-	1201	1101.8	99.2	1829	727.2
5-Chloro-	1206	1101.8	104.2	1966	864.2
6-Chloro-	1214	1101.8	112.2	1947	845.2
3,4-Dichloro-	1434	1347.4	86.6	2426	1078.6
3,5-Dichloro-	1388	1347.4	40.6	2314	966.6
3,6-Dichloro-	1387	1347.4	39.6	2160	812.6
4,5-Dichloro-	1358	1347.4	10.6	2036	688.6
4,6-Dichloro-	1326	1347.4	−21.4	1957	609.6
5,6-Dichloro-	1376	1347.4	38.6	2193	845.6
3,4,5-Trichloro-	1577	1593.0	−16.0		
3,4,6-Trichloro-	1529	1593.0	−67.0		
3,5,6-Trichloro-	1522	1593.0	−70.0		
4,5,6-Trichloro-	1510	1593.0	−82.0		
Tetrachloro-	1722	1838.5	−116.5		

hyde group causes a reduction, whereas separation of the groups considerably increases the values.

Chlorination of the two hydroxybenzaldehydes has opposite effects. Reduced I^* values occur in both series, but when the two substituents are together, *i.e.*, in 2-hydroxybenzaldehyde, chlorination has the least effect adjacent to the hydroxy group, *i.e.*, at the 3-, the 3,4- and the 3,4,5-positions. Mono-, di- and trichlorination

TABLE VII

RETENTION (I), DISPERSION (I_M) AND SELECTIVITY (I)* INDICES OF CHLORINATED AROMATIC COMPOUNDS ON SE-30

Parameter	*Compound*			
	Phenol	*Benzaldehyde*	*o-Hydroxybenzaldehyde*	*p-Hydroxybenzaldehyde*
I	964	978	1062	1320
I_M	656.6	742.2	856.3	856.3
I^*	307.4	235.8	205.7	463.7
Monochlorination I^*:				
Maximum	4-Chloro	–	3-Chloro-	2-Chloro-
Minimum	2-Chloro-	–	4-Chloro-	3-Chloro-
Dichlorination I^*:				
Maximum	3,5-Dichloro-	–	3,4-Dichloro-	2,6-Dichloro-
Minimum	2,4-Dichloro- 2,5-Dichloro-	–	4,6-Dichloro-	2,5-Dichloro-
Trichlorination I^*:				
Maximum	3,4,5-Trichloro-	–	3,4,5-Trichloro-	2,3,6-Trichloro-
Minimum	2,3,5-Trichloro-	–	4,5,6-Trichloro-	2,3,5-Trichloro-

of 4-hydroxybenzaldehyde gives a much greater selectivity value, with a smaller effect of the two separated groups. On chlorination, a smaller effect occurs adjacent to the aldehyde group, *i.e.*, 2-chloro-, 2,6-dichloro- and 2,3,6-trichlorohydroxy.

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MICRODETERMINATION OF 11-DEHYDROTHROMBOXANE B_2 IN HUMAN URINE BY GAS CHROMATOGRAPHY–SELECTED-ION MONITORING[a]

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SUMMARY

Simple and effective purification for quantitation of trace amounts of 11-dehydrothromboxane B_2 (11-dehydro-TXB_2) in human urine by gas chromatography–selected-ion monitoring (GC–SIM) was achieved. The procedure is based on stepwise elution of the methyl ester derivative from a silica gel column with *n*-hexane–ethyl acetate (1:1) after washing the column with *n*-hexane–ethyl acetate (2:1). After the methyl ester has been converted into the corresponding dimethylisopropylsilyl ether, GC–SIM is carried out by monitoring the ion at *m/z* 539.32 for 11-dehydro-TXB_2 and that at *m/z* 543.33 for its $^{18}O_2$-labelled variant as an internal standard. The detection limit is 2 pg per injection with a signal-to-noise ratio of 5:1. The method was applied to the determination of 11-dehydro-TXB_2 in human urine.

INTRODUCTION

Thromboxane A_2 (TXA_2), one of the cyclooxygenase products of arachidonic acid, is a potent vasoconstrictor and platelet activator. In order to measure the TXA_2 production *in vivo*, thromboxane B_2 (TXB_2) has been widely used as a stable hydrolysis product. However, as the level of TXB_2 in plasma is readily confounded by platelet activation *ex vivo*, the measurement of the plasma concentration of this compound does not reflect exactly the circulating TXA_2 level. In response to this

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significant problem, attention has been focused on searching for more stable metabolites as indices of thromboxane biosynthesis. Thus, 11-dehydro-TXB_2, which is one of the major metabolites of TXB_2 in plasma and urine[1,2], has been considered to reflect the TXA_2 release in the human circulation[3,4]. In addition, the urinary excretion of 11-dehydro-TXB_2 is higher than that of the other TXA_2 metabolites, such as 2,3-dinor-TXB_2 and TXB_2[5,6]. Several methods for the microdetermination of 11-dehydro-TXB_2 in biological specimens by using radioimmunoassay or gas chromatography–selected-ion monitoring (GC–SIM) have been reported[1,3,5]. These methods require complicated clean-up by thin-layer and/or high-performance liquid chromatographic (HPLC) techniques in order to eliminate interfering substances in biological specimens. We have reported that purification by silica gel column chromatography after methylation of prostaglandins (PGs) with ethereal diazomethane is very effective for the determination of PGs by GC–SIM[7,8]. This method was applied to the purification of 11-dehydro-TXB_2 in urine. This paper deals with a convenient method for the determination of 11-dehydro-TXB_2 in urine without the use of tedious purification procedures and/or special mass spectrometric techniques.

EXPERIMENTAL

Sample and reagents

11-Dehydro-TXB_2 was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Dimethylethylsilyl(DMES)-, dimethyl-*n*-propylsilyl(DMnPS)- and dimethylisopropylsilyl(DMiPS)imidazoles were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Pierce (Rockford, IL, U.S.A.). Sephadex LH-20 and silica gel were obtained from Pharmacia (Uppsala, Sweden) and E. Merck (Darmstadt, F.R.G.). A Chem-Elut cartridge was purchased from Analytichem International (Harbor City, CA, U.S.A.). A Sep-Pak C_{18} cartridge was purchased from Waters Assoc. (Milford, MA, U.S.A.). Other solvents and reagents used were of the highest quality available.

Capillary gas chromatography

A Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector and data processing system was employed. An open-tubular capillary column (Ultra 1, 25 m × 0.3 mm I.D.) (Hewlett-Packard, Avondale, PA, U.S.A.) was used. Helium was used as the carrier gas and make-up gas. An all-glass VandenBerg-type solventless injector[9] was used for sample injection. The temperature of the injection port and detector was kept at 320°C and that of the column oven at 280°C.

Gas chromatography–mass spectrometry

A Hitachi (Ibaragi, Japan) M-80B gas chromatograph–mass spectrometer equipped with an electron-ionization source and data processing system and a VG (Manchester, U.K.) 70-SE gas chromatograph–mass spectrometer equipped with an electron-ionization source and data processing system were employed. An Ultra 1 open-tubular capillary column (25 m × 0.3 mm I.D.) was used. The flow-rate of the carrier gas was maintained at 10 ml/min. An inlet pressure of 0.1 kg/cm^2 produced a linear gas velocity of 25 cm/s. The temperature of the column oven was kept at 280°C, the injector at 320°C and the ionization source at 200°C. The ionization energy and

accelerating voltage were 20 eV and 3 kV for the Hitachi M-80B and 35 eV and 8 kV gor the VG 70-SE, respectively. SIM was carried out with a mass spectrometric resolution of 3000, 4000 and 12 000 ($M/\Delta M$) by monitoring the mass number of the ion corresponding to a particular elemental composition.

Preparation of ^{18}O-labelled 11-dehydro-TXB_2

^{18}O-labelled 11-dehydro-TXB_2 was prepared according to a modification of the procedure described by Strife and Murphy[10]. Ethereal diazomethane (2 ml) was added to a solution of 11-dehydro-TXB_2 (0.2 mg) in methanol (0.2 ml), and the resulting solution was allowed to stand at room temperature for 30 min. The reaction mixture was evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in 0.2 *M* $Li^{18}OH$ (0.1 ml) and the resulting solution was sonicated for 2 h. The solution was acidified to pH 2 with 0.5 *M* hydrochloric acid and then extracted with ethyl acetate. The organic layer was washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate and evaporated. The above operation was repeated five times. The final product was dissolved in acetone (0.2 mg/ml) and stored at −20°C.

Derivatization procedure

To a solution of 11-dehydro-TXB_2 in methanol (0.1 ml) was added freshly prepared ethereal diazomethane (0.5 ml), and the resulting solution was allowed to stand at room temperature for 1 h. After evaporation of the solvent, the residue was silylated with BSTFA, DMES-, DMnPS- and DMiPS-imidazole (20 μl). The resulting mixture was kept at 60°C for 1 h, then the excess of the reagent except for BSTFA was removed by Sephadex LH-20 column chromatography[11,12].

Collection and storage of urine samples

Urine was collected over a period of 24 h. During the collection period, the samples were kept in a refrigerator. The volume was determined and the urine was stored at −40°C until assayed.

Extraction and purification of 11-dehydro-TXB_2 from human urine

After addition of the ^{18}O-labelled 11-dehydro-TXB_2 (5 ng) as an internal standard, human urine (1–5 ml) was acidified to pH 2 with 0.5 *M* hydrochloric acid, allowed to stand at room temperature for 1 h and then transferred to a Chem-Elut column (No. 1003, 1005 or 1010). 11-Dehydro-TXB_2 was eluted with ethyl acetate (24–50 ml) and the eluate was evaporated to dryness. The residue was dissolved in 15% aqueous ethanol (10 ml) and acidified to pH 2 with 0.5 *M* hydrochloric acid. The resulting solution was applied to a Sep-Pak C_{18} cartridge, and the cartridge was washed with 15% aqueous ethanol (10 ml) and *n*-hexane (10 ml). 11-Dehydro-TXB_2 was eluted with ethyl acetate (10 ml). This eluate was evaporated to dryness and the residue was dissolved in methanol (0.5 ml). To this solution freshly prepared ethereal diazomethane (2 ml) was added, and the mixture was allowed to stand at room temperature for 1 h. After evaporation of the solvent, the residue was dissolved in *n*-hexane–ethyl acetate (2:1) (3 ml) and then transferred to a silica gel column (5 × 0.5 cm I.D.). The column was washed with *n*-hexane–ethyl acetate (2:1) (8 ml) and then the methyl ester of 11-dehydro-TXB_2 was eluted with *n*-hexane–ethyl acetate (1:1) (20 ml).

The eluate was evaporated to dryness and the residue was silylated with DMiPS-imidazole as described above.

RESULTS AND DISCUSSION

Gas chromatography–mass spectrometry

In the GC–MS analysis of prostaglandins (PGs) and thromboxanes (TXs), the corresponding methyl ester (ME)–alkyloxime (RO)–dimethylalkylsilyl (DMAS) ether derivatives have been widely used. The TMS, DMES, DMnPS and DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester were prepared by treatment with ethereal diazomethane and then with the corresponding silylating reagents. The resulting derivatives were used for the investigation of their GC–MS properties. Each of the reaction products exhibited a well shaped, single gas chromatographic peak. When analysed on a methylsilicone cross-linked fused-silica capillary column, the methylene unit values of these 11-dehydro-TXB_2 derivatives increased in the order TMS, DMES, DMnPS, and DMiPS ether derivatives, as listed in Table I.

The mass spectra of the TMS, DMES, DMnPS and DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester are shown in Fig. 1. The mass fragmentation patterns of the DMES, DMnPS and DMiPS ether derivatives were closely related to that of the TMS ether derivative, except for a 14 or 28 a.m.u. shift for each hydroxy group. For instance, the fragment ion of $[M-71]^+$, produced by cleavage of the C-15–C-16 bond, implies the presence of two silanoxy bonds by the shift from *m/z* 455 in the TMS ether derivative to *m/z* 483 (455 + 14 × 2) in the DMES ether derivative and to *m/z* 511 (455 + 28 × 2) in the DMnPS and DMiPS ether derivatives. In general, an increase in the carbon number of the silylating agent has a tendency to yield the $[M-alkyl]^+$ ion with high abundance in the high-mass region[13]. Of these DMAS ether derivatives, the relative intensities of the $[M-alkyl]^+$ ions in the DMES and DMnPS ether derivatives of 11-dehydro-TXB_2 ME were not increased in comparison with that of the $[M-CH_3]^+$ ion in the corresponding TMS ether derivative, whereas the DMiPS ether derivative gave the $[M-C_3H_7]^+$ ion as a base peak and more than 10% of total ion current was concentrated in this base-peak ion. The appearance of the base peak in the high-mass region may be useful for the specific and sensitive detection of 11-dehydro-TXB_2 in biological specimens by GC–SIM.

The mass spectrum of the corresponding DMiPS ether derivative exhibited the series of ions which were determined to be characteristic of the expected structure. The molecular ion was not observed. The loss of the isopropyl radical from a DMiPS group in the molecular ion gave rise to the ion of $[M-43]^+$ at *m/z* 539 as a base peak,

TABLE I

METHYLENE UNIT VALUES OF TMS, DMES, DMnPS AND DMiPS ETHER DERIVATIVES OF 11-DEHYDRO-TXB_2 METHYL ESTER

Derivative	*Methylene unit value*
TMS	28.16
DMES	31.18
DMnPS	32.33
DMiPS	32.55

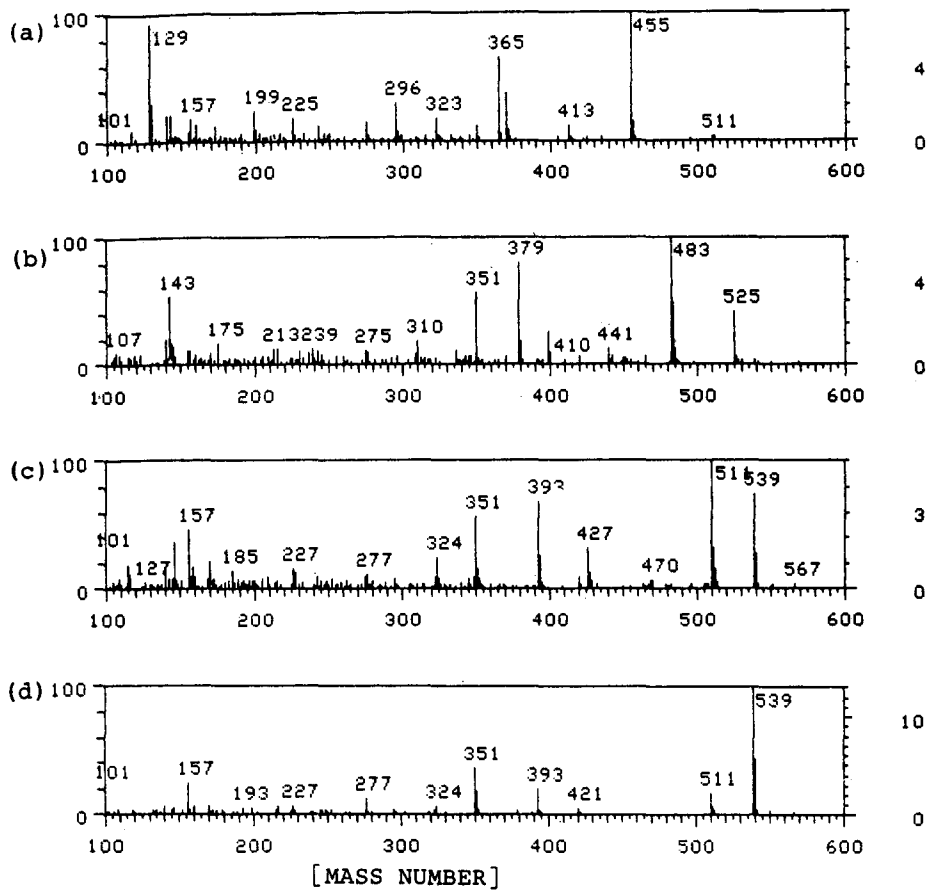
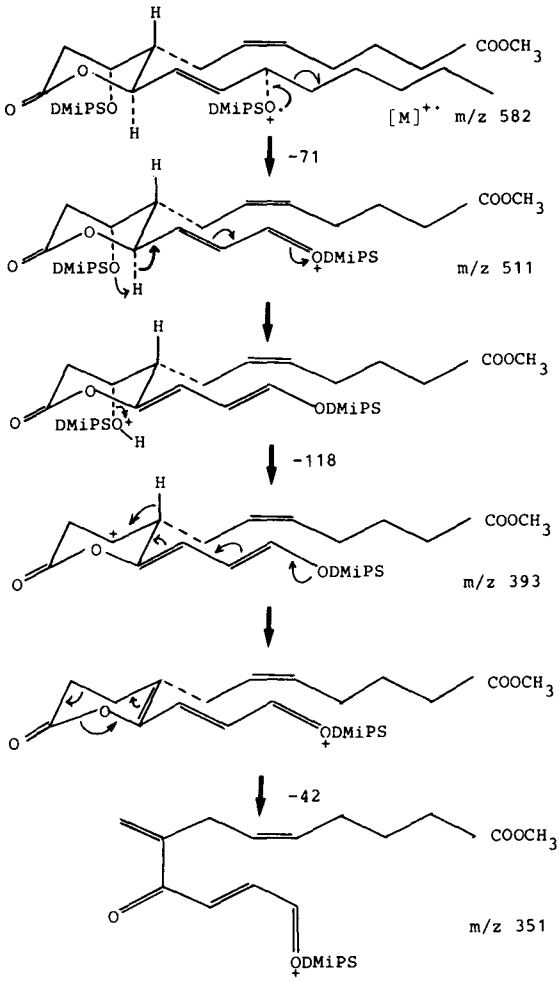


Fig. 1. Mass spectra of the (a) TMS, (b) DMES, (c) DMnPS and (d) DMiPS ether derivatives of 11-dehydro-TXB_2 methyl ester.

indicating the incorporation of two DMiPS groups into the 11-dehydro-TXB_2 methyl ester. This ion produced the ion at *m/z* 421 by the loss of dimethylisopropylsilanol (DMiPSOH: 118 a.m.u.). The radical loss of a C_{16}–C_{20} hydrocarbon fragment from the molecular ion, which is a typical mass fragmentation of prostanoids, produced the characteristic ion at *m/z* 511. This ion produced the ion at *m/z* 393 by the loss of a DMiPSOH molecule, initiated by a migration of the C-9 DMiPSO group to the hydrogen atom at C-12. The ion at *m/z* 393 further fragmented to that at *m/z* 351 by the migration of the hydrogen atom at C-8 to C-9, followed by the loss of a CH_2O group, as shown in Scheme 1. The resulting mass spectral data revealed that the ME–DMAS ether derivatives were in the lactone form.

In order to examine the applicability of the ME–DMiPS ether derivative of 11-dehydro-TXB_2, selected-ion monitoring was carried out, using the characteristic ion of $[M-43]^+$ at *m/z* 539 with a mass spectral resolution of 4000. The detection limit of this derivative was about 2 pg per injection with a signal-to-noise ratio of 5:1.



Scheme 1.

Internal standard of ^{18}O-labelled 11-dehydro-TXB$_2$

Fig. 2 shows the mass spectrum of the ^{18}O-labelled 11-dehydro-TXB$_2$ methyl ester–bis-DMiPS ether derivative, which is closely related to that of the corresponding non-labelled 11-dehydro-TXB$_2$ derivative, except for the obvious shift produced by the substitution of oxygen-18 atoms. The mass spectrometric analysis revealed that the product was a mixture of multi-^{18}O-labelled 11-dehydro-TXB$_2$ and the enrichments of the $^{18}O_3$-, $^{18}O_2$- and $^{18}O_1$-labelled variants were found to be 47.7, 43.6 and 8.5%, respectively. On the other hand, the content of the non-labelled 11-dehydro-TXB$_2$ derivative was calculated to be less than 0.3%.

Calibration graph

The calibration graph for 11-dehydro-TXB$_2$ was obtained by plotting the peak-area ratio of 11-dehydro-TXB$_2$ to an internal standard against their weight

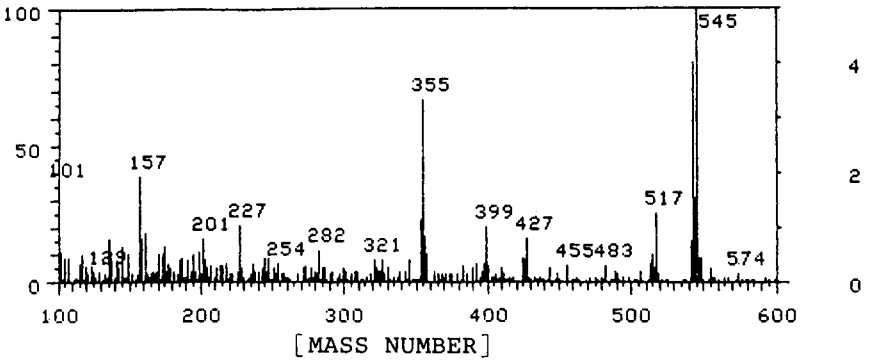


Fig. 2. Mass spectrum of ^{18}O-labelled 11-dehydro-TXB_2 methyl ester–bis-DMiPS ether derivative.

ratios. Good linearity was observed between the peak-area ratio and weight ratio of 11-dehydro-TXB_2 and its ^{18}O-labelled variant in the range 0–100 pg.

pH-dependent equilibrium between the lactone and open forms of 11-dehydro-TXB_2

It has been reported that there is a pH-dependent equilibrium between the lactone form of 11-dehydro-TXB_2 and its open form, and that the open form is obtained more easily at a higher pH, but the lactone is slowly formed at a lower pH[1]. Therefore, a time course study of this pH-dependent equilibrium between the open and lactone forms of 11-dehydro-TXB_2 was carried out to test the validity of the use of the ^{18}O-labelled variant as an internal standard, because the endogenous 11-dehydro-TXB_2 in human urine is presumably in the open form whereas the ^{18}O-labelled variant is in the lactone form.

The following urine samples were prepared. (1) A human urine sample was adjusted to pH 9 with 1 *M* sodium hydroxide solution and allowed to stand for 3 h at room temperature. After the above urine sample had been adjusted to pH 2 with 0.5 *M* hydrochloric acid, an aliquot of the ^{18}O-labelled variant was added to the sample as

TABLE II

TIME COURSE OF THE pH-DEPENDENT EQUILIBRIUM BETWEEN THE OPEN AND LACTONE FORMS OF 11-DEHYDRO-TXB_2

Experiment	*Lactone ring-opening conditions*			*Lactonization conditions*			*Found (ng/ml)*
	Addition of I.S.[a]	*pH*	*Standing time (h)*	*Addition of I.S.*[a]	*pH*	*Standing time (h)*	
I_1	−	9.0	3	+	2.0	1	0.683
I_2	−	9.0	3	+	2.0	3	0.686
I_3	−	9.0	3	+	2.0	Overnight	0.638
II_1	+	9.0	3	−	2.0	1	0.649
II_2	+	9.0	3	−	2.0	3	0.626
II_3	+	9.0	3	−	2.0	Overnight	0.673
III_1	−	−	−	+	2.0	1	0.662

[a] I.S. = internal standard (^{18}O-labelled 11-dehydro-TXB_2).

an internal standard. The resulting sample was allowed to stand for 1 h, 3 h and overnight. (2) An aliquot of the internal standard was added to the human urine prior to sample preparation. The urine sample was adjusted to pH 9 with 1 *M* sodium hydroxide solution and allowed to stand for 3 h at room temperature. The sample was adjusted to pH 2 with 0.5 *M* hydrochloric acid and allowed to stand for 1 h, 3 h and overnight at room temperature. (3) The urine sample to which the internal standard had been added was adjusted to pH 2 with 0.5 *M* hydrochloric acid and allowed to stand for 1 h at room temperature.

The determination of 11-dehydro-TXB_2 in the above urine samples was carried out. There was no statistically significant difference in the amounts of 11-dehydro-TXB_2 in these samples, as shown in Table II. This indicates that the formation of the lactone from the open form proceeds rapidly. This finding is different from the results of a time course study reported previously[1].

Sample preparation

The purification of the prostanoids was performed by silica gel column chromatography with ethyl acetate–methanol (99:1) as eluent, as described in previous papers[8,14]. This method was applied to the purification of 11-dehydro-TXB_2 from human urine, but it was difficult to find the peak of 11-dehydro-TXB_2 owing to interference of endogenous substances with the selected-ion recording. Therefore, an HPLC purification step was added to the above procedure before methylation, according to the method of Powell[15]. However, the separation of the peak in the selected-ion recording was not improved, as shown in Fig. 3a. The peak of 11-dehydro-TXB_2 could be observed by the use of temperature programming, holding the temperature at 200°C for 1 min and then increasing it at 5°C/min to 300°C, and high-resolution SIM ($M/\Delta M$ = 12 000), as shown in Fig. 3b.

The above facts required the development of a novel procedure for the purification of 11-dehydro-TXB_2 for GC–SIM. Schweer *et al.*[5] and Chiabrando *et al.*[6] have pointed out the importance and necessity of purification for the quantitation of trace amounts of prostanoids in biological specimens, even when an MS–MS technique was used. Therefore, the pattern of elution of 11-dehydro-TXB_2 methyl ester from a silica gel column was examined in detail by stepwise elution, using mixtures of *n*-hexane and ethyl acetate in various ratios[16]. This experiment showed that the 11-dehydro-TXB_2 methyl ester was more lipophilic than the other prostaglandins, such as $PGF_2\alpha$ and PGE_2, and was eluted with *n*-hexane–ethyl acetate (1:1) after washing the column with *n*-hexane–ethyl acetate (2:1). There was little interfering material in this fraction. The reproducibility of the elution of the 11-dehydro-TXB_2 methyl ester from a silica gel column was extremely good, and the mean and standard deviation of the recovery of 11-dehydro-TXB_2 from human urine through the present purification procedure were 72.6 ± 3.5% (n = 5).

Fig. 4 shows a typical selected-ion recording, obtained by analysing an aliquot of the urine extract. The peaks appearing in this selected-ion recording correspond to *ca.* 10 pg of 11-dehydro-TXB_2 and *ca.* 25 pg of its $^{18}O_2$-labelled variant, used as an internal standard. The selcted-ion recording was obtained with an extremely good signal-to-noise ratio when the ion monitored was $[M-43]^+$ at m/z 539.32 for the ME–DMiPS ether derivative of 11-dehydro-TXB_2 and at m/z 543.33 for its $^{18}O_2$-labelled variant at a mass spectrometric resolution of 4000. The interfering

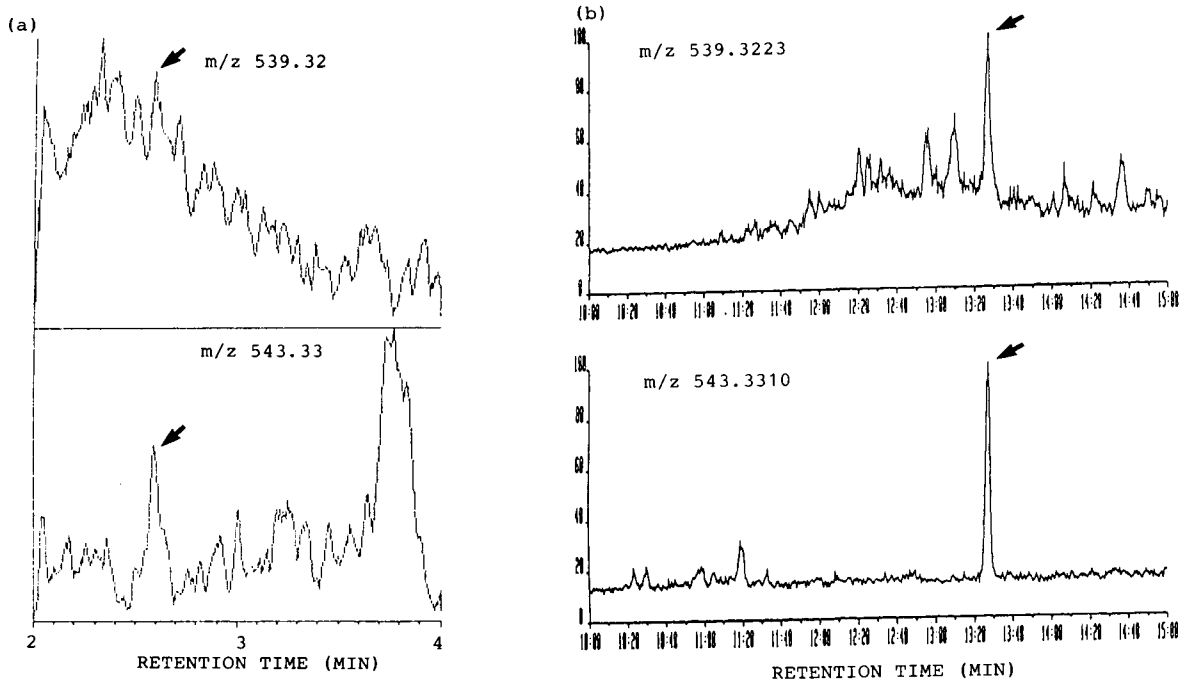


Fig. 3. Selected-ion recordings of human urinary 11-dehydro-TXB_2, purified by HPLC, monitoring the characteristic ion of $[M-43]^+$ in (a) a low-resolution mode ($M/\Delta M = 3000$) and (b) a high-resolution mode ($M/\Delta M = 12\,000$) with temperature-programmed GC.

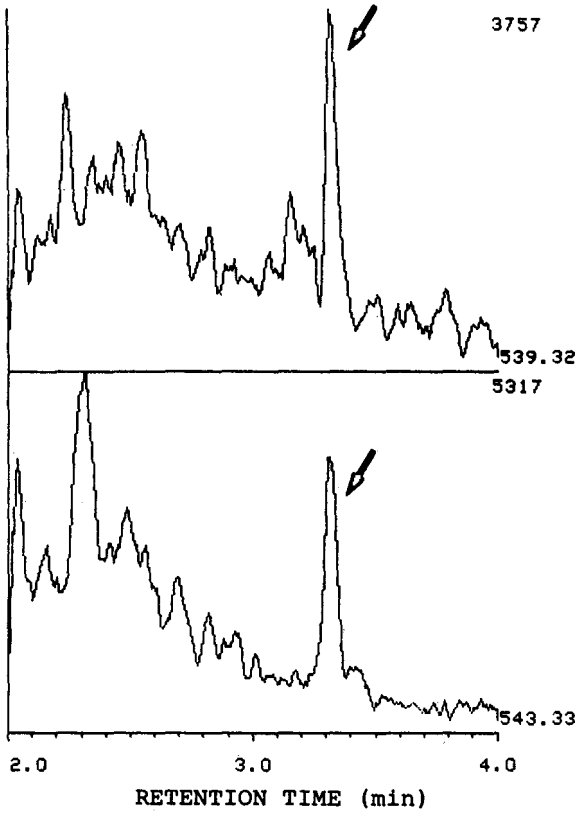


Fig. 4. Selected-ion recordings of the ME–DMiPS ether derivatives of 11-dehydro-TXB_2 (*m/z* 539.32) and its $^{18}O_2$-labelled variant (*m/z* 543.33) in the extract from human urine, monitoring the characteristic ion of $[M-43]^+$ at a mass spectrometric resolution of 4000.

substances in the extract from the urine were almost eliminated by the new purification procedure.

Reproducibility

In order to examine the accuracy and precision of the present method, four urine samples spiked with 11-dehydro-TXB_2 at concentrations of 0.25, 0.50, 0.75 and 1.00 ng/ml were prepared. These concentrations corresponded to approximately 50, 100, 150 and 200% of endogenous 11-dehydro-TXB_2, found in the urine of the healthy male volunteers. The results are given in Table III. The mean and standard deviation of the recovery of 11-dehydro-TXB_2 added were 101.5 $\pm$ 2.4% ($n = 8$). Statistical analysis was carried out according to a two-way layout in order to divide the analytical errors between the three sources of sample preparation, 11-dehydro-TXB_2 concentrati on and GC–SIM. The analysis indicates that there was no significant difference among sample preparation and 11-dehydro-TXB_2 concentration, and that almost all of the total error in this experiment could be attributed to the GC–SIM, because the errors between sample preparation and 11-dehydro-TXB_2 concentration were negligible. The coefficient of variation in GC–SIM was 4.5%.

TABLE III

RECOVERY OF 11-DEHYDRO-TXB_2 ADDED TO HUMAN URINE

Sample	*Added (ng/ml urine)*	*Found (ng/ml urine)*		*Mean recovery (%)*
1a	0	0.525	0.484	—
1b	0	0.515	0.562	
2a	0.25	0.786	0.844	104.1
2b	0.25	0.840	0.765	
3a	0.50	1.084	0.968	99.5
3b	0.50	1.016	1.016	
4a	0.75	1.290	1.218	99.5
4b	0.75	1.303	1.268	
5a	1.00	1.597	1.544	103.3
5b	1.00	1.579	1.581	

According to the orthogonal polynomial equation, the estimated urinary levels and their 95% confidence limit were calculated to be 0.527 ± 0.073, 0.544 ± 0.106 and 0.090 ± 0.033 ng/ml, and were in good agreement with the concentrations of endogenous 11-dehydro-TXB_2 in non-spiked urine (0.522, 0.557 and 0.084 ng/ml, respectively), as shown in Table IV. These facts suggest that the present method makes it possible to determine picogram levels of 11-dehydro-TXB_2 in urine with high reliability. The urinary levels of 11-dehydro-TXB_2 in normal subjects and patients are under investigation and these results will be discussed elsewhere.

TABLE IV

DETERMINATION OF 11-DEHYDRO-TXB_2 IN HUMAN URINE

Subject	*Urine volume (ml per 24 h)*	*11-Dehydro-TXB_2 concentration (ng/ml)*		*Urinary excretion (μg per 24 h)*
		Present method (mean)	*Orthogonal polynomial equation method (mean ± 95% confidence limit)*	
A	1120	0.522	0.527 ± 0.073	0.590
B	745	0.557	0.544 ± 0.106	0.405
C	2810	0.084	0.090 ± 0.033	0.253

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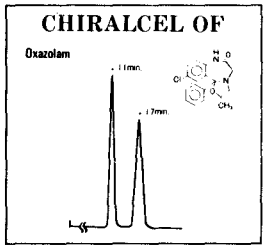
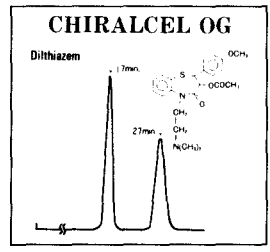
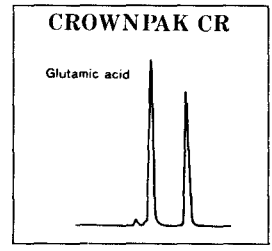
Carbinoxamine	1.39	OD
Carteolol	1.86	OD
Chlophedianol	2.82	OJ
Chlormezanone	1.47	OJ
Cyclopentolate	2.47	OJ
Diltiazem	1.46	OD
	2.36	OF
	1.75	OG
Disopyramide	2.46	OF
Ethiazide	1.54	OF
Ethotoin	1.40	OJ
Fenoprofen	1.35	OJ
Glutethimide	2.48	OJ

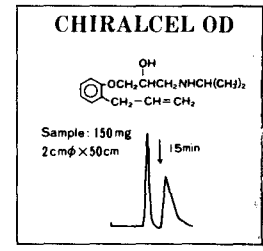
SUBSTANCE	α	column
Gauifenesin	2.40	OD
Hexobarbital	1.7	CA-1
Homatropine	3.13	OD
Hydroxyzine	1.17	OD
Indapamide	1.58	OJ
Ketamine	complete resolution	CA-1
Ketoprofen	1.46	OJ
Mephobarbital	5.9	OJ
	2.3	CA-1
Methaqualone	2.8	CA-1
	7.3	OJ
Methsuximide	2.68	OJ
Metoprolol	complete resolution	OD
Mianserin	1.75	OJ
Nilvadipine	complete resolution	OT

SUBSTANCE	α	column
Oxapadol	complete resolution	CA-1
Oxazepan	4.36	OC
Oxazolam	1.67	OF
Oxprenolol	6.03	OD
Perisoxal	1.33	OF
	1.27	OD
Pindolol	5.07	OD
Piprozolin	1.7	CA-1
Praziquantal	complete resolution	CA-1
Propranolol	2.29	OD
Rolipram	complete resolution	CA-1
Sulconazole	1.68	OJ
Suprofen	1.6	OJ
Trimebutine	1.81	OJ
Warfarin	1.96	OC









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