ISSN 0021-9673

VOL. 629 NO. 1 JANUARY 15, 1993

Symp. on Solid-Phase Extraction in Environmental and Clinical Chemistry, San Francisco, CA, April 5–10, 1992

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

ELSEVIER

JOURNAL OF CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

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

Submission of Papers. The preferred medium of submission is on disk with accompanying manuscript (see *Electronic manuscripts* in the Instructions to Authors, which can be obtained from the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, Netherlands). Manuscripts (in English; *four* copies are required) should be submitted to: Editorial Office of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, Netherlands, Telefax (+31-20) 5862 304, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, Netherlands. Review articles are invited or proposed in writing to the Editors who welcome suggestions for subjects. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications*) has 40 volumes in 1993. The subscription prices for 1993 are:

J. Chromatogr. (incl. *Cum. Indexes, Vols. 601–650*) + *Biomed. Appl.* (Vols. 612–651):
Dfl. 8520.00 plus Dfl. 1320.00 (p.p.h.) (total *ca.* US$ 5927.75)

J. Chromatogr. (incl. *Cum. Indexes, Vols. 601–650*) only (Vols. 623–651):
Dfl. 7047.00 plus Dfl. 957.00 (p.p.h.) (total *ca.* US$ 4821.75)

Biomed. Appl. only (Vols. 612–622):
Dfl. 2783.00 plus Dfl. 363.00 (p.p.h.) (total *ca.* US$ 1895.25).

Subscription Orders. The Dutch guilder price is definitive. The US$ price is subject to exchange-rate fluctuations and is given as a guide. Subscriptions are accepted on a prepaid basis only, unless different terms have been previously agreed upon. Subscriptions orders can be entered only by calender year (Jan.–Dec.) and should be sent to Elsevier Science Publishers, Journal Department, P.O. Box 211, 1000 AE Amsterdam, Netherlands, Tel. (+31-20) 5803 642, Telefax (+31-20) 5803 598, or to your usual subscription agent. Postage and handling charges include surface delivery except to the following countries where air delivery via SAL (Surface Air Lift) mail is ensured: Argentina, Australia, Brazil, Canada, China, Hong Kong, India, Israel, Japan*, Malaysia, Mexico, New Zealand, Pakistan, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. *For Japan air delivery (SAL) requires 25% additional charge of the normal postage and handling charge. For all other countries airmail rates are available upon request. Claims for missing issues must be made within six months of our publication (mailing) date, otherwise such claims cannot be honoured free of charge. Back volumes of the *Journal of Chromatography* (Vols. 1–611) are available at Dfl. 230.00 (plus postage). Customers in the USA and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 655 Avenue of the Americas, New York, NY 10010, USA, Tel. (+1-212) 633 3750, Telefax (+1-212) 633 3764.

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

US Mailing Notice. *Journal of Chromatography* (ISSN 0021-9673) is published weekly (total 58 issues) by Elsevier Science Publishers (Sara Burgerhartstraat 25, P.O. Box 211, 1000 AE Amsterdam, Netherlands). Annual subscription price in the USA US$ 5927.75 (subject to change), including air speed delivery. Application to mail at second class postage rate is pending at Jamaica, NY 11431. **USA POSTMASTERS:** Send address changes to *Journal of Chromatography*, Publications Expediting, Inc., 200 Meacham Avenue, Elmont, NY 11003. Airfreight and mailing in the USA by Publication Expediting.

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

0021-9673/93/$06.00

Printed in the Netherlands

For Contents, see p. VII

JOURNAL OF CHROMATOGRAPHY

VOL. 629 (1993)

JOURNAL of CHROMATOGRAPHY

INCLUDING ELECTROPHORESIS AND OTHER SEPARATION METHODS

SYMPOSIUM VOLUMES

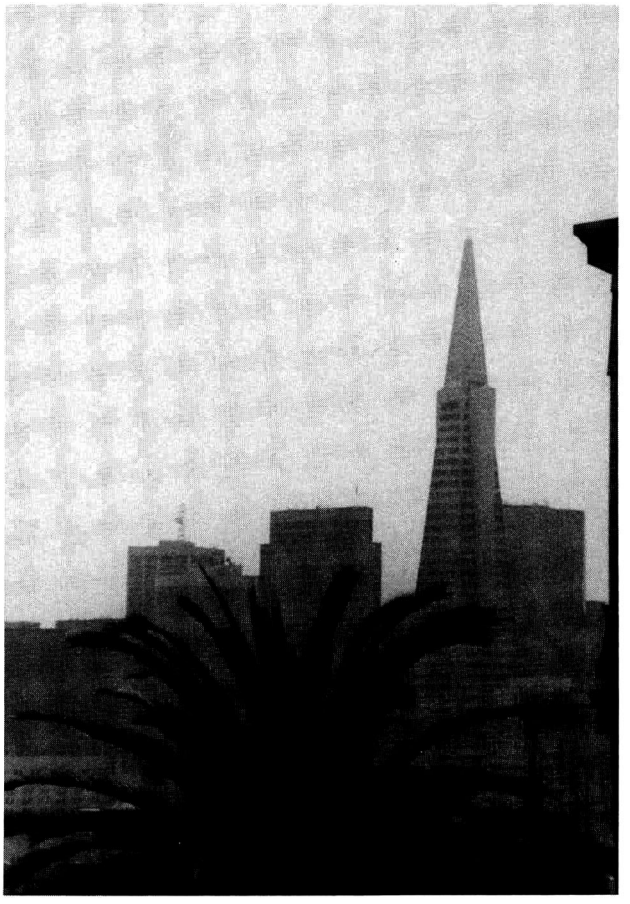
ELSEVIER
AMSTERDAM — LONDON — NEW YORK — TOKYO

J. Chromatogr., Vol. 629 (1993)

0021-9673/93/$06.00

Printed in the Netherlands

SYMPOSIUM ISSUE



SYMPOSIUM ON SOLID-PHASE EXTRACTION IN ENVIRONMENTAL AND CLINICAL CHEMISTRY

HELD IN CONJUNCTION WITH THE 203RD ACS NATIONAL MEETING

San Francisco, CA (USA), April 5–10, 1992

Guest Editors

M. S. MILLS
(Lawrence, KS)

E. M. THURMAN
(Lawrence, KS)

VOL. 629 NO. 1 JOURNAL OF CHROMATOGRAPHY JANUARY 15, 1993

CONTENTS

SYMPOSIUM ON SOLID-PHASE EXTRACTION IN ENVIRONMENTAL AND CLINICAL CHEMISTRY, SAN FRANCISCO, CA, APRIL 5–10, 1992

Journal of Chromatography, 629 (1993) 1
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 776

Foreword

This thematic issue, *Solid-Phase Extraction in Environmental and Clinical Chemistry*, consists of a collection of papers presented at the *203rd American Chemical Society Meeting* in San Francisco, CA, April 1992. The papers discuss some of the many facets of sample preparation using solid-phase extraction and serve as important reference points in this rapidly expanding technology. The symposium organizers thank the Environmental Division of the American Chemical Society (ACS) for their support of the symposium and to the *Journal of Chromatography* for the compilation of these research papers.

Solid-phase extraction has presented a superior alternative to liquid–liquid extraction during the past decade for analyte purification and concentration in many analytical methods. Solid-phase extraction continues to evolve into one of the most effective methods for the isolation of target analytes from complex mixtures. Moreover, solid-phase extraction provides cleaner extracts prior to sensitive instrument analysis, such as gas chromatography–mass spectrometry, high-performance liquid chromatography, infrared spectroscopy, nuclear magnetic resonance spectroscopy and immunoassay. With today's demands for rapid analysis of multiple samples at low cost and the increasing advancements in automation technology, solid-phase extraction has a secure place in the future of sample-preparation technology, providing reproducible results with minimal technician requirements.

Lawrence, KS (USA) Margaret S. Mills
E. Michael Thurman

Journal of Chromatography, 629 (1993) 3–9
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 580

Variables affecting the supercritical fluid extraction of analytes from octadecylsilane solid-phase sorbents

Kenneth G. Furton and Ethon Jolly

Department of Chemistry, Florida International University, University Park, Miami, FL 33199 (USA)

Joseph Rein

Department of Chemistry, Florida International University, University Park, Miami, FL 33199 (USA) and Toxicology Laboratory, Dade County Medical Examiner's Office, Miami, FL 33136 (USA)

ABSTRACT

The relative effects of the numerous variables which can influence the supercritical fluid extraction (SFE) efficiencies of analytes from solid-phase extraction (SPE) sorbents is discussed. SFE efficiencies are not only influenced by experimental variables such as temperature and pressure (density), but also by sample/matrix variables, extraction vessel variables and collection variables. The significance of many of these variables are underrated, and studies aimed at quantitative comparisons of these variables have generally focused on extraction of analytes from endogenous matrices, rather than SPE sorbents such as those evaluated here. The relative effects of temperature and density have been quantitatively compared for the extraction of polycyclic aromatic hydrocarbons and methoxychlor from octadecylsilane sorbents. Under the conditions studied, the effect of temperature was found to be of equal importance to that of density, and either could be used to vary the recovery of analytes over an extremely wide range. A more thorough knowledge of the relative effects of all of the controllable variables should facilitate optimization of SFE of analytes from SPE sorbents for maximum selectivity as well as maximum overall recoveries.

INTRODUCTION

Solid-phase extraction (SPE) has rapidly established itself as an important sample preparation technique for both matrix simplification and trace enrichment in a variety of clinical and environmental applications. One of the most commonly used SPE sorbents is the reversed-phase octadecylsilane sorbent used in numerous clinical and environmental applications, including the analysis of drugs, essential oils, food preservatives, vitamins, plasticizers, pesticides, steroids, hydrocarbons, toxins, etc. [1–7]. SPE techniques have numerous advantages over liquid–liquid extraction techniques, including ease of automation, reduced cost, reduced solvent use and higher sample throughput. However, SPE techniques still suffer from numerous problems, particularly when applied to the analysis of biological specimens [3]. The chemical background from impurities, contaminants, antioxidants, etc., often observed when using SPE, can interfere in the subsequent analysis of the sample, as well as reduce the lifetime of chromatographic columns [8–10]. Running blanks and cleaning of the cartridges to minimize interferences diminishes sample throughput and adds to solvent consumption and processing costs. Many of these problems can potentially be minimized by the use of highly selective elution solvents which can elute the analyte of interest while

Correspondence to: K. G. Furton, Department of Chemistry, Florida International University, University Park, Miami, FL 33199, USA.

0021-9673/93/$06.00

leaving the matrix interferences. Supercritical fluids have unique solvent properties which may make them ideal candidates for selective elution of target analytes without increased solvent consumption or reduced sample throughput.

Supercritical fluid extraction (SFE) has proven to be a powerful alternative to conventional liquid extraction methods, such as Soxhlet extraction, particularly in environmental applications [11,12]. Most of the applications which have emerged recently have involved the extraction of analytes directly from endogenous solid and liquid matrices, although the gaseous trapping of analytes onto various adsorbents with subsequent recovery by SFE has also been investigated [11–13]. Solid-phase sorbents have been used in combined SFE–supercritical fluid chromatography (SFC) techniques where the sorbent acts as a chromatographic column with selective elution of target analytes [14–16]. Alternatively, solid-phase sorbents have been used as efficient traps for off-line SFE [17]. In this paper, we discuss the potential for the use of supercritical fluids as elution solvents to enhance the selectivity, as well as overall recovery, of analytes trapped onto SPE sorbents. Subsequent SFE of analytes can be used on-line, combined with gas, liquid or supercritical fluid chromatography, to provide a complete sample extraction and analysis system, or, more commonly, can be employed off-line with sorbent or solvent trapping of the analytes. On-line methods provide the greatest sensitivity and, in principle, should be more accurate due to the reduced sample handling. Off-line methods often allow the greatest experimental flexibility and the possibility of multiple analysis of the extract regardless of the sample conditions. For these reasons, off-line methods are the most common.

Supercritical fluids possess unique physicochemical properties which make them attractive as alternative extraction solvents to liquids currently used. Supercritical fluids have low viscosities and zero surface tension which allows for very efficient penetration into macroporous materials such as those used in SPE. Additionally, the significantly higher diffusivities of solutes in supercritical fluids provides for more rapid transport out of the sorbent bed. The greatest advantage of supercritical fluids, however, is the fact that they have densities (and often solvating powers) comparable to that of liquids, which can be continuously varied by as much as an order of magnitude by varying the temperature and pressure of the extraction vessel. This paper continues a systematic, experimental investigation of the effects that controllable variables have on the supercritical fluid elution of analytes from SPE sorbents. This work focuses on the off-line solvent collection of polycyclic aromatic hydrocarbons (PAHs) and methoxychlor eluted from octadecylsilane sorbents, and a discussion of the major variables affecting achievable recoveries by this method.

EXPERIMENTAL

All standards were obtained from Aldrich (Milwaukee, WI, USA) and used without further purification. PrepSep (Fisher Scientific, Orlando, FL, USA) octadecylsilane (C_{18}) solid phase extraction cartridges were used in this study. A stock packing containing 200 ppm of the standards on C_{18} was prepared by slow evaporation of a standard chloroform solution. The SFE apparatus used has been described previously [18]. SFE-grade carbon dioxide (Scott Specialty Gases, Plumsteadville, PA, USA) was used for all of the extractions. In order to accurately quantify the effects of temperature and density (pressure at constant temperature), care was taken to ensure that all of the other major controllable experimental variables were kept constant and precisely measured. Numerous extractions were performed and only those extractions with the same flow-rates (0.60 ml/min), extraction times (5.3 min) and total volumes (3.2 ml of liquid carbon dioxide) were compared for temperature and density effects. Flow-rates were controlled by varying the length of linear restrictors fabricated from 40 μm I.D. x 375 μm O.D. fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA). Extractions were performed on packings contained in a 1.0 x 1.0 cm I.D. vessel. Extraction conditions were purposely chosen to yield less than quantitative recovery of the analytes to allow for comparisons of the different extraction conditions. Analytes were collected into methylene chloride with fluorene added as an external standard. Identification and quantitation of the analytes were performed using a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a flame ionization detection.

RESULTS AND DISCUSSION

The elution of analytes from SPE sorbents is controlled by a variety of interrelated factors, including the affinity of the analytes for the sorbent, the vapor pressure of the analytes and the solubility and diffusion coefficient of the analytes in the supercritical fluid. In addition, SFE efficiencies are controlled by a complex relationship between many experimental variables, some of which are listed in Table I. Although it is well established that, to a first approximation, the solvent power of a supercritical fluid is related to its density, the relative effects of many of the other controllable SFE variables are poorly understood. The list in Table I was tabulated from observations in the authors' laboratory for the SFE of analytes from octadecylsilane sorbents. Some of the relative effects observed for octadecylsilane sorbents are different from those seen for other SPE sorbents and may be different from those seen for the extraction of analytes from endogenous solid matrices as discussed below. Much additional work is needed to distinguish which of these variables are significant and their relative effects for the multitude of possible analyte–sorbent(matrix) combinations. The variables affecting SFE of analytes from sorbents can be broken down into four main areas, namely, experimental variables, sample/matrix variables, extraction vessel variables and, finally, collection variables. Each group from Table I is discussed in more detail below.

Experimental variables

The experimental variables are those which can be continuously varied during an extraction to maximize selectivity, as well as overall recoveries. These variables often have the largest effect on observed recoveries and will be discussed first. Carbon dioxide is the primary fluid used in most SFE applications because it has low critical points (T_c = 31.3°C, P_c = 1070 p.s.i.), is non-toxic, non-flammable, odorless, readily available in high purity, inexpensive and eliminates solvent waste disposal problems. Unfortunately, carbon dioxide has one severe problem; it is non-polar and, therefore, of limited value for very polar or ionic analytes. There are three possible approaches to overcoming the polarity problem of carbon dioxide. First, one can choose a more polar supercritical fluid (*e.g.* $CHClF_2$, N_2O), although at the expense of many of the advantages stated above [19]. A second approach, which is more commonly used, involves the addition of a small volume percent of an organic solvent modifier to increase the solvent strength/selectivity of the carbon dioxide. A third approach, which can be combined with pure or modified carbon dioxide SFE, is to chemically derivatize the analyte *in situ* to a more extractable form. In this approach, the analyte is simultaneously derivatized and extracted by static SFE, followed by dynamic extraction and analyte collection. This approach has been applied with success to the extraction of a variety of analytes directly from environmental ma-

TABLE I

VARIABLES WHICH MAY AFFECT THE SUPERCRITICAL FLUID ELUTION OF ANALYTES FROM SPE SORBENTS EMPLOYING OFF-LINE SOLVENT COLLECTION

Experimental variables	Sample/matrix variables	Extraction vessel variables	Collection variables
Primary fluid	Sorbent type	Extraction vessel dimensions	Solvent type
Fluid modifiers	Analyte type	Extraction vessel size	Solvent temperature
Static SFE/derivatization	Sorbent condition	Extraction vessel dead volume	Solvent volume
Density (pressure)	Weight of sorbent	Extraction vessel orientation	Fluid flow-rate
Temperature	Analyte concentration		
Total volume of extraction fluid	Co-extractants present		
Fluid flow-rate	Sorbent particle size		
Extraction time			

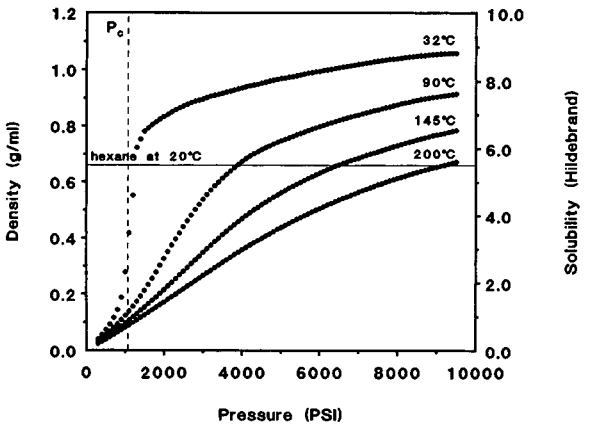


Fig. 1. Plot of carbon dioxide density (and calculated Hildebrand solubility parameter) *versus* temperature and pressure. The density of hexane at 20°C is shown for comparison.

trices as well as from analytes collected on Empore C_{18} sorbent disks [20].

The next controllable experimental variables which can have tremendous effects on achievable recoveries are density (at a constant temperature) and temperature (at a constant density). The temperature is an extremely powerful variable, often underrated in optimization schemes. Typical densities and Hildebrand solubility parameters for supercritical carbon dioxide are shown in Fig. 1 at various temperatures and pressures (data calculated with SF Solver software, Isco, Lincoln, NE, USA), illustrating the extremely wide range of solubilities available at a variety of temperatures. The relative effect of these two variables are illustrated later in this paper for the elution of PAHs and methoxychlor from octadecylsilane SPE sorbents. In general, maximum recoveries are achieved by maximizing these two variables, although careful optimization of temperature and density allows for pre-elution of potential SPE contaminants and highly selective elution of analytes [14,18]. The extraction of analytes by deposition onto SPE sorbents, followed by elution with supercritical fluids, can be called SFC since chromatographic processes are involved in the selectivity of the extraction, although, experimentally, this technique more closely resembles SFE. The final two controllable experimental variables are the extraction time and fluid flow-rate, which yield the total elution volume of supercritical fluid. For the analytes studied here, we have generally found that the total volume of the extraction fluid used is more important than the extraction time. It is important to note that this is the opposite of the effect reported for the SFE of environmental solids where kinetic effects may dominate and the total extraction time is often one of the most important variables [21].

Sample/matrix variables

Sample/matrix variables can be extremely important in achievable elution of analytes from solid-phase sorbents. The type of analyte and the type of sorbent/matrix will often dictate the feasibility of quantitative recovery by SFE. In addition, the particle size and the condition of the sorbent (moisture content, pH, etc.), as well as the concentration of the analyte and any co-extractants present can significantly affect observed recoveries. Finally, the weight of sorbent extracted can affect SFE recoveries and relates to other experimental and extraction vessel variables, such as the total volume of extraction fluid used and/or the total extraction time, as well as extraction cell dead volume at a constant extraction cell size. These sample/matrix variables are often dictated by the SPE conditions chosen and are often more difficult to adjust for maximizing selectivity/overall recoveries using supercritical fluids.

Extraction vessel variables

The third set of variables in Table I relate to the design of the extraction vessel. We have previously published the significant effect that the dimensions of the extraction vessel (I.D. to length) can have on the elution of PAHs and methoxychlor from octadecylsilane SPE sorbents [18,22]. These results are different than those observed for the SFE of analytes directly from environmental solids where no effect has been seen [23]. A more thorough discussion of the effect of cell dimensions and comparisons for different SPE matrix/analyte types has recently been published [24]. The size of the extraction vessel combined with the sample size determine the amount of dead volume within the vessel. In some cases, for the SFE of PAHs from octadecylsilane supports, we have observed increases in recoveries when dead volume was present in the extraction

vessel compared to when it was completely filled. Again, this is in conflict with observations for the SFE of analytes directly from environmental solids where lower recoveries have been observed when significant dead volume is present [25]. Clearly, the relative effects of SFE variables for the recovery of analytes from endogenous matrices cannot be directly applied to predict relative effects of SFE of solid-phase sorbents. The orientation of the extraction vessel (vertical or horizontal) may also affect observed recoveries depending on whether the supercritical fluid enters the top or the bottom of the vessel (in the vertical orientation) and if there is dead volume in the vessel. Since these variables generally have only small effects on achievable recoveries and may actually decrease reproducibility, prudent practice is to always completely fill the extraction vessel.

Collection variables

The final variables which must be considered are those related to the final trapping of the extracted analyte in the supercritical fluid. One of the most common forms of analyte collection is to decompress the supercritical fluid directly into a liquid solvent. Improper control of collection variables can result in losses improperly attributed to poor SFE efficiencies [23]. The analytes must have a high solubility in the chosen collection solvent and a sufficient solvent volume (*e.g.* > 1 ml) must be present to efficiently trap the target analytes. The solvent temperature should be maintained at a low enough temperature to prevent volatilization losses of analytes while at a high enough temperature to prevent freezing of the solvent (due to the cooling effect from the rapid expansion of the supercritical carbon dioxide) or blocking of the restrictor. Finally, the flow-rate of the supercritical fluid is often limited to *ca.* < 2 ml/min (and ideally < 1 ml/min) due to the purging and solvent disruption encountered with solvent trapping at very high flow-rates. Purging losses can become significant particularly for more volatile analytes using long extraction times. The ideal collection conditions are dependent on the analyte and co-extractants. For non-volatile analytes, such as the 4–6 ring PAHs and methoxychlor studied here, 1 ml of methylene chloride was adequate to quantitatively trap these analytes.

Relative effects of temperature and density

Data for the supercritical carbon dioxide elution of methoxychlor, pyrene, perylene and benzo[*ghi*]-perylene from octadecylsilane sorbents are given in Table II. It is important to note that conditions were chosen to deliberately yield recoveries of at least 1% and less than 100% for these analytes to allow for a quantitative relative comparison between these two variables. Quantitative recoveries

TABLE II

RECOVERY OF ANALYTES FROM OCTADECYL-BONDED PACKINGS USING VARIOUS DENSITIES AT VARIOUS TEMPERATURES AND DENSITIES

Variables			Recovery of analytes (%) (average S.D. = 1.2)			
CO_2 volume (ml)	Density (g/ml)	Temperature (°C)	Methoxychlor	Pyrene	Perylene	Benzo[*ghi*]perylene
7.5	0.70	40.0	37.6	22.1	1.9	0.2
7.5	0.70	50.0	69.1	39.2	7.4	1.9
7.5	0.70	60.0	75.2	60.1	13.7	4.1
7.5	0.70	80.0	94.3	78.7	24.1	7.8
7.5	0.70	100.0	102.1	80.9	47.8	27.4
3.0	0.40	100.0	25.4	26.6	5.6	0.6
3.0	0.50	100.0	38.8	41.4	9.2	2.6
3.0	0.60	100.0	59.0	50.8	14.6	6.6
3.0	0.70	100.0	80.7	70.2	22.2	4.0

of these analytes were readily achieved by maximizing the temperature and/or density of the supercritical fluid. Selectivity tuning of supercritical fluid elution of analytes, however, requires a quantitative measure of the relative effects of each controllable variable. Recoveries increased in direct proportion to the density of supercritical carbon dioxide (at a constant temperature of 100°C) whereas there was an approximately linear logarithmic increase in recovery with the inverse of the temperature (at a constant density of 0.70). Linear least squares regression analysis of these data yields the following equations:

$$\text{Recovery}_{\text{methoxychlor}} = 186.1d - 51.38 \quad (r^2 = 0.99) \quad (1)$$

$$\text{Recovery}_{\text{methoxychlor}} = \exp[5.336 - 63.25(t^{-1})] \quad (r^2 = 0.92) \quad (2)$$

$$\text{Recovery}_{\text{pyrene}} = 140.5d - 29.99 \quad (r^2 = 0.98) \quad (3)$$

$$\text{Recovery}_{\text{pyrene}} = \exp[5.429 - 89.43(t^{-1})] \quad (r^2 = 0.95) \quad (4)$$

$$\text{Recovery}_{\text{perylene}} = 55.20d - 17.46 \quad (r^2 = 0.97) \quad (5)$$

$$\text{Recovery}_{\text{perylene}} = \exp[5.902 - 204.4(t^{-1})] \quad (r^2 = 0.98) \quad (6)$$

$$\text{Recovery}_{\text{benzo[}ghi\text{]perylene}} = 33.90d - 13.57 \quad (r^2 = 0.98) \quad (7)$$

$$\text{Recovery}_{\text{benzo[}ghi\text{]perylene}} = \exp[6.236 - 301.4(t^{-1})] \quad (r^2 = 0.96) \quad (8)$$

where d = density and t = temperature.

Using the above equations, it is possible to compare the maximum effect of temperature and density on recoveries of these analytes. The data, as well as the curves generated from the above equations, are shown in Fig. 2 for perylene and pyrene over a density range of 0.20 g/ml (1500 psi at 100°C) to 0.90 g/ml (10 000 p.s.i. at 100°C) and just above the critical temperature, 32°C, to 142°C. It is obvious that the effect of temperature is as great as, or in some cases greater than, that of density over the ranges compared. The great selectivity of this technique is apparent when one considers that recoveries can be increased from 0 to 100% simply by varying either the temperature or the density of the

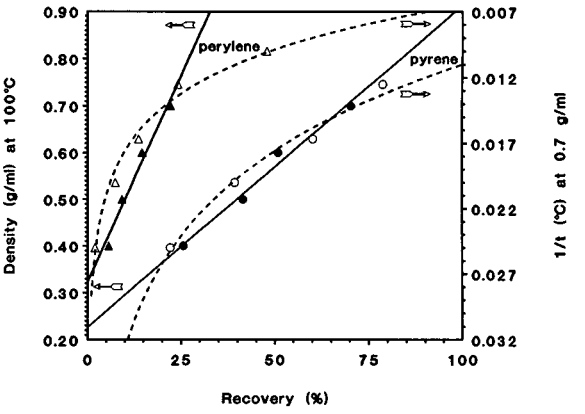


Fig. 2. Plot of the actual recoveries and calculated recoveries of perylene and pyrene from octadecysilane sorbents as a function of supercritical carbon dioxide density (solid lines) and inverse of the temperature (dashed lines).

supercritical carbon dioxide. The effect of temperature is sometimes underrated in SFE optimization schemes and appeared to be particularly important when employing supercritical fluids as SPE elution solvents. Increasing the supercritical fluid temperature (at a constant density) generally enhances the solubility, vapor pressure and the diffusion coefficient of the analyte in the supercritical fluid, as well as reducing the affinity of the analyte for sorptive sites on the SPE matrix.

CONCLUSIONS

The use of supercritical fluids for the selective extraction of compounds from solid-phase sorbents has great potential. Current limitations arise from our limited understanding of relative effects and interrelationships between the large number of experimental variables controlling the supercritical fluid elution of analytes from SPE sorbents, as well as the relatively expensive instrumentation required for SFE. As our understanding of these variables increases, our ability to fine tune SFE for highly selective extractions, as well as maximum overall recoveries should increase dramatically. In addition, the ease of automation of SFE techniques and reduced solvent consumption could potentially make supercritical fluids cost-effective alternatives to conventional liquid solvents. The use of supercritical fluids in combination with SPE will likely have a bright

future due to the following potential advantages of supercritical fluids over conventional solvents: (1) highly selective pre-elution of interferents by simple changes in temperature/density of the supercritical fluid; (2) more selective extraction of target analytes from matrix; (3) straightforward on-line/chromatographic analysis can increase recoveries, with decreased analysis times; (4) rapid simultaneous derivatization/extraction possible; (5) methodology may be directly applied to some samples with minimal sample preparation (*e.g.* biological tissues) with the potential for developing completely automated SFE–SPE(SFC)–SFE methods.

ACKNOWLEDGEMENT

Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support of this research.

REFERENCES

1 C. F. Poole and S. K. Poole, *Chromatography Today*, Elsevier, Amsterdam, 1991.
2 I. D. Wilson, E. D. Morgan and S. J. Murphy, *Anal. Chim. Acta*, 236 (1990) 145.
3 K. G. Furton and J. Rein, *Anal. Chim. Acta*, 236 (1990) 99.
4 J. M. Vinuesa, J. C. M. Cortes, C. I. Canas and G. F. Perez, *J. Chromatogr.*, 472 (1989) 365–370.
5 G. A. Junk and J. J. Richard, *Anal. Chem.*, 60 (1988) 451.
6 O. Suzuki, H. Seno and T. Kumazawa, *J. Forensic Sci.*, 33 (1988) 1249.
7 M. J. Shepherd, M. Holmes and J. Gilbert, *J. Chromatogr.*, 354 (1986) 305.
8 G. A. Junk, M. J. Avery and J. J. Richard, *Anal. Chem.*, 60 (1988) 1347.
9 H. D. Rood, *Anal. Chim. Acta*, 236 (1990) 115.
10 W. E. Johnson, N. J. Fendinger and J. R. Plimmer, *Anal. Chem.*, 63 (1991) 1510.
11 S. B. Hawthorne, *Anal. Chem.*, 62 (1990) 633A.
12 V. Lopez-Avila, N. S. Dodhiwala and W. F. Beckert, *J. Chromatogr. Sci.*, 28 (1990) 468.
13 J. M. Wong, N. Y. Kado and P. A. Kuzmicky, *Anal. Chem.*, 63 (1991) 1644.
14 J. M. Levy, R. A. Cavalier, T. N. Bosch, A. F. Rynaski and W. E. Huhak, *J. Chromatogr. Sci.*, 27 (1989) 341.
15 E. D. Ramsey, J. R. Perkins and D. E. Games, *J. Chromatogr.*, 464 (1989) 353.
16 J. E. France, J. W. King and J. M. Snyder, *J. Agric. Food Chem.*, 39 (1991) 1871.
17 L. J. Mulcahey, J. L. Hendrick and L. T. Taylor, *Anal. Chem.*, 63 (1991) 2225.
18 J. Rein, C. M. Cork and K. G. Furton, *J. Chromatogr.*, 545 (1991) 149.
19 S. B. Hawthorne, J. J. Langenfeld, D. J. Miller and M. D. Burford, *Anal. Chem.*, 64 (1992) 1614.
20 S. B. Hawthorne, D. J. Miller, D. E. Nivens and D. C. White, *Anal. Chem.*, 64 (1992) 405.
21 K. D. Bartle, A. A. Clifford, S. B. Hawthorne, J. J. Langenfeld, D. J. Miller and R. Robinson, *J. Supercrit. Fluids*, 3 (1990) 143.
22 K. G. Furton and J. Rein, *Chromatographia*, 31 (1991) 297.
23 J. J. Langenfeld, M. D. Burford, S. B. Hawthorne and D. J. Miller, *J. Chromatogr.*, 594 (1992) 297.
24 K. G. Furton and Q. Lin, *Chromatographia*, 34 (1992) in press.
25 S. B. Hawthorne, personal communication.

Journal of Chromatography, 629 (1993) 11–21
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 671

Application of mixed-mode, solid-phase extraction in environmental and clinical chemistry

Combining hydrogen-bonding, cation-exchange and Van der Waals interactions

M. S. Mills and E. M. Thurman

Water Resources Division, US Geological Survey, 4821 Quail Crest Place, Lawrence, KS 66049-3839 (USA)

M. J. Pedersen

Clinical Reference Laboratory, 11850 W. 87th Street, Lenexa, KS 66214 (USA)

ABSTRACT

Silica- and styrene–divinylbenzene-based mixed-mode resins that contain C_8, C_{18} and sulphonated cation-exchange groups were compared for their efficiency in isolation of neutral triazine compounds from water and of the basic drug, benzoylecgonine, from urine. The triazine compounds were isolated by a combination of Van der Waals and hydrogen-bonding interactions, and benzoylecgonine was isolated by Van de Waals interactions and cation exchange. All analytes were eluted with a polar organic solvent containing 2% ammonium hydroxide. Larger recoveries (95%) were achieved on copolymerized mixed-mode resins where C_{18} and sulfonic acid are in closer proximity than on "blended" mixed-mode resins (60–70% recovery).

INTRODUCTION

Solid-phase extraction (SPE) is a sample-preparation tool used for the isolation, concentration, and purification of analytes from complex matrices such as serum, urine, saliva, tissue, food, and contaminated water. Reversed-phase (C_2, C_4, C_8 and C_{18}), cation- and anion-exchange, and various polar phases (silica, alumina, and cyano phases) are used in SPE. The technique of SPE has wide-range application in drug-screening laboratories, environmental-monitoring programs, and the food and cosmetic industry. An expansive literature on the subject of SPE has developed during the last 10 years [1,2].

Recently, various phases of SPE have been blended or copolymerized in order to use multiple interactions for isolation and purification of analytes, called mixed-mode resins [3–6]. These resins have the potential to recover analytes covering a wider-range of polarity by utilizing specific, simultaneous interactions. Furthermore, elution steps can be selective by sequentially cancelling specific mechanisms of interaction in operation on one resin. Prior to the advent of mixed-mode resins, there had been many reports on mixed-mode interactions in high-performance liquid chromatography (HPLC) [7–10] and in SPE resins [11–14]. For example, silica-based HPLC columns and SPE resins show mixed-mode interactions between active silanol sites and

Correspondence to: M. S. Mills, Water Resources Division, US Geological Survey, 4821 Quail Crest Place, Lawrence, KS 66049, USA.

0021-9673/93/$06.00

polar functional groups of the organic analyte, especially for basic analytes containing nitrogen. However, the utilization of such interactions for enhanced isolation of more polar compounds has been realized only recently [13,14].

To capitalize on the duality of isolation mechanisms, either multiple functionalities are bonded onto a common frame (copolymerized), or different resins are blended into one cartridge. Although some papers have dealt with the tandem use of resins [15,16], few papers have dealt with the merits and drawbacks of mixed-mode resins [3,4] or have discussed the diversity of mixed-mode resins presently available. Therefore, the objectives of this paper are to: (1) present current research on the mechanisms of mixed-mode interactions for two suites of analytes, triazine herbicides and drugs of abuse, and (2) compare and contrast the efficiency of isolation of "bonded" and "blended" mixed-mode resins using both environmental and clinical compounds as examples.

EXPERIMENTAL

Reagents

Methanol (Burdick & Jackson, Muskegon, MI, USA), ethyl acetate, and isooctane (Fisher Scientific, Springfield, NJ, USA) were the pesticide-grade solvents used. Deionized water was charcoal filtered and glass distilled prior to use. The triazine herbicides ametryn, atrazine, prometon, prometryn, propazine, simazine and terbutryn were obtained from Supelco (Bellefonte, PA, USA), and the triazine metabolites, 2-amino-4-chloro-6-ethylamino-*s*-triazine and 2-amino-4-chloro-6-isopropylamino-*s*-triazine, were obtained from Ciba Geigy (Greensboro, NC, USA). The benzoylecgonine and [$^2H_3$]benzoylecgonine were obtained from Sigma (St. Louis, MO, USA) and pentafluoropropanol and pentafluoropropionic anhydride were obtained from Alltech (Deerfield, IL, USA). The MP-3 mixed-mode resin (Interaction Chromatography, Mountain View, CA, USA), which included C_{18} chains and sulfonic acid groups (0.8 mequiv./g), contained 100 mg of 45-μm particles of styrene–divinylbenzene. The Bond-Elut Certify[a] mixed-mode resin (Varian Sample Preparation, Harbor City, CA, USA) contained 300 mg of 40-μm particles of silica which included either C_{18} chains or sulfonic acid groups (1 mequiv./g). All sulfonic acid groups were in hydrogen-saturated form. Standard solutions were prepared in methanol. [$^2H_{10}$]Phenanthrene (US Environmental Protection Agency, Cincinnati, OH, USA) was used as an internal standard for gas chromatography–mass spectrometry (GC–MS) quantification of the triazine herbicides.

Solid-phase extraction

SPE cartridges for the triazine herbicides were preconditioned sequentially with 2 ml methanol and 2 ml distilled water on a Millilab workstation by the robotic probe (Waters, Milford, MA, USA). Samples were passed by positive pressure through the cartridge at a flow-rate of less than 2.0 ml/min as greater flow-rates were not physically possible due to back pressure. The herbicides were eluted by the workstation with 4 ml ethyl acetate, which contained 2% ammonium hydroxide, and the eluate was robotically spiked with 500 μl of internal standard [$^2H_{10}$]phenanthrene (0.2 ng/μl). Approximately 100 μl of water trapped in the cartridge were coeluted with the ethyl acetate. The ethyl acetate and water layers were mixed after addition of the internal standard, and after settling, 3.5 ml of the homogenized ethyl acetate layer were drawn off the denser water layer by the robotic probe into a new centrifuge tube. Eluates were evaporated down to 100 μl by a Turbovap (Zymark, Hopkinton, MA, USA) at 45°C under a nitrogen stream and transferred to a glass-lined vial for GC–MS analysis.

SPE cartridges for benzoylecgonine were preconditioned with 2 ml of methanol followed by 2 ml of 0.1 *M* phosphate buffer at pH 6.0 on a vacuum manifold (Supelco, Bellefonte, PA, USA). A 1-ml volume of sample was spiked with 1000 ng of [$^2H_3$]benzoylecgonine in 100 μl of deionized water. Then, 2.0 ml of 0.1 *M* phosphate buffer at pH 6.0 were added, and the sample was passed through the cartridge under medium vacuum. The sample was washed with 3.0 ml of deionized water, 3.0 ml of 0.1 *M* HCl, and 9.0 ml of methanol. The cartridge was eluted with 2.0 ml of methylene chloride–isopropanol (80:20, v/v) with 2% ammonium hydroxide. The sample was then evaporated to dryness and derivatized by adding 50 μl of pentafluoropropionic

[a] The use of trade names in this paper is for identification purposes only and does not constitute endorsement by the US Geological Survey.

anhydride and 25 μl of pentafluoropropanol to a screw-cap test tube and heating at 75°C for 15 min. The sample was evaporated to dryness at 50°C under nitrogen and taken up in 100 μl of ethyl acetate for GC–MS analysis.

GC–MS analysis

Automated GC–MS analysis of the eluates were performed on a Hewlett-Packard Model 5890 GC (Palo Alto, CA, USA) and a 5970A mass selective detector. Operating conditions for the triazine herbicides and benzoylecgonine were as follows: ionization voltage, 70 eV; an ion source temperature of 250°C, electron multiplier, 2200 V; direct capillary interface at 280°C, tuned daily with perfluorotributylamine; and a 50-ms dwell period. Separation of the herbicides and benzoylecgonine was accomplished with a fused-silica capillary column of methyl silicone (HP-1) with a film thickness of 0.33 μm, 12 m × 0.2 mm I.D. (Hewlett-Packard). Helium was used as the carrier gas at a flow-rate of 1 ml/min and a head pressure of 35 kPa. The column temperature was held at 50°C for 1 min, then ramped at 6°C/min to 250°C where it was held for 10 min. Injector temperature was 280°C.

The filament and multiplier were not turned on until 5 min into the analysis. Quantification of the base peak of each triazine was based on the response of the 188 (amu) ion of the internal standard, [$^2H_{10}$]phenanthrene. Confirmation of the compound was based on the presence of the molecular ion and two confirming ions with a retention-time match of ± 0.2% relative to [$^2H_{10}$]phenanthrene and correct area ratios for confirming ions. All triazine herbicides were selectively monitored. Benzoylecgonine was monitored with selected ions of 300, 316, and 421 u; and the [$^2H_3$]benzoylecgonine was monitored with 303, 319, and 424 u ions. Quantification of benzoylecgonine was by internal standard using the [$^2H_3$]benzoylecgonine.

Adsorption isotherm experiments

Adsorption isotherms for the triazine herbicides and metabolites were performed in triplicate on MP-3 and MP-1 resins from distilled water. SPE cartridges (containing 100 mg of packing) were preconditioned sequentially with 3 ml methanol and 3 ml distilled water. The cartridge was cut open, and the resin emptied into a PTFE-lined glass vial containing 40 ml distilled water. Analytes were spiked into the water from individual 1-mg/ml stock solutions in methanol. The samples were allowed to equilibrate for 24 h on a mechanical shaker, and the resin was allowed to settle. Approximately 35 ml of clear water sample were pipetted off into a clean, weighed test tube to determine the exact volume of water present. The sample then was passed through a disposable 0.45-μm filter (Millipore Filtrex) and preconditioned C_{18} cartridge (Waters Millipore) (conditioned sequentially with 3 ml methanol, 3 ml ethyl acetate, 3 ml methanol and 3 ml distilled water) at 20 ml/min [17]. The cartridge was eluted with 3 ml ethyl acetate, and the eluate was spiked with 500 μl of internal standard, [$^2H_{10}$]phenanthrene (0.2 ng/μl). The ethyl acetate layer was drawn off the co-eluted, denser water layer and evaporated to 100 μl for transfer to a glass-lined vial for analysis by GC–MS with selective ion monitoring.

RESULTS AND DISCUSSION

Mixed-mode isolation of s-triazine compounds

To understand the mechanism of isolation of triazine herbicides on a mixed-mode resin, it is first necessary to discuss the structure of the suite of analytes. Fig. 1 shows the structure of the two dealkylated metabolites and the parent *s*-triazine suite. Common to each member of the suite is a triazine ring, with variations in the length of the alkyl side chains at the 4′ and 6′ positions and the moiety at the 2′ position. The 2′ position is occupied either by a chlorine atom (deethylatrazine, deisopropylatrazine, atrazine, simazine and propazine), a methylthio group (ametryn, prometryn and terbutryn), or a methoxy linkage (prometon). Primary amine moieties are present on the dealkylated metabolites, and secondary amines are present on all parent triazine herbicides. Retention of chlorine-containing triazine herbicides is reported to follow structural changes on the reversed-phase C_{18} resins, with capacity of the herbicides increasing in a linear logarithmic fashion with the addition of methylene groups to the alkyl side chain [18], which indicates Van der Waals interactions. Deisopropylatrazine, which contains only two carbon atoms in alkyl side chains, is retained the least on C_{18}, and propazine, which contains six carbon atoms, is retained the most.

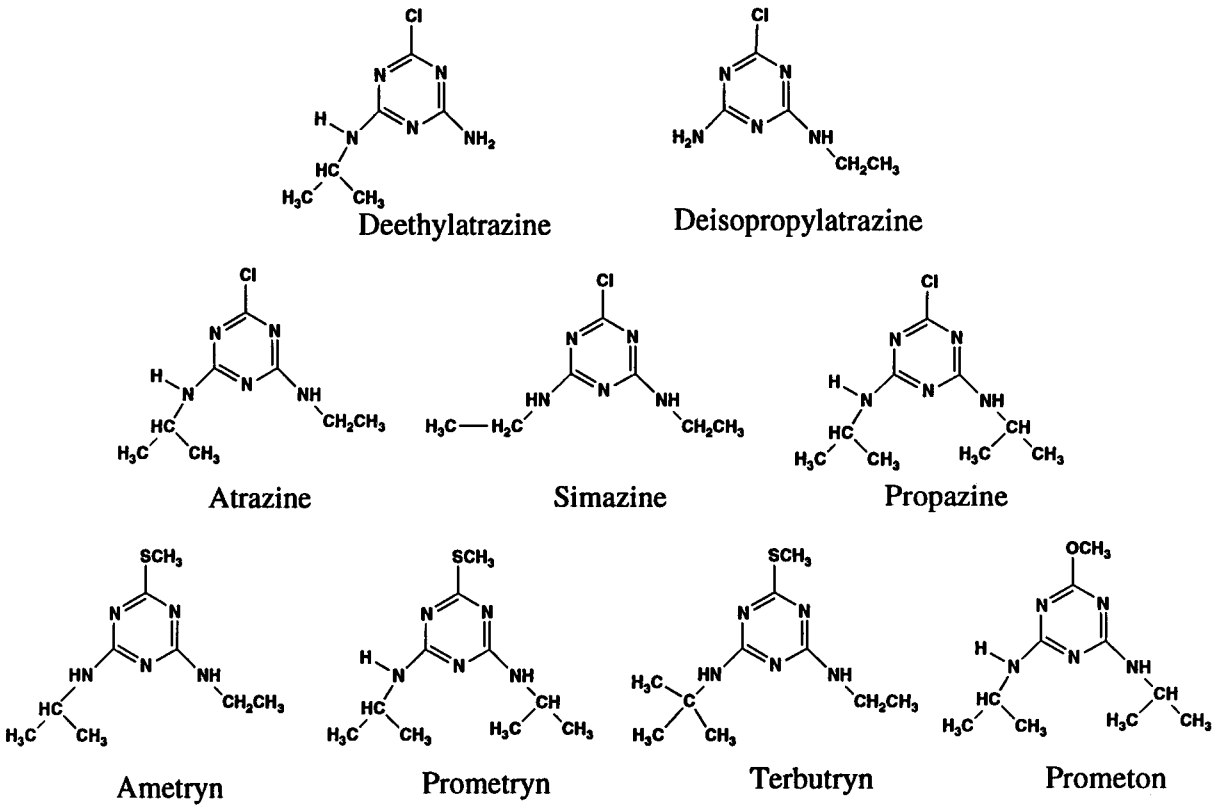


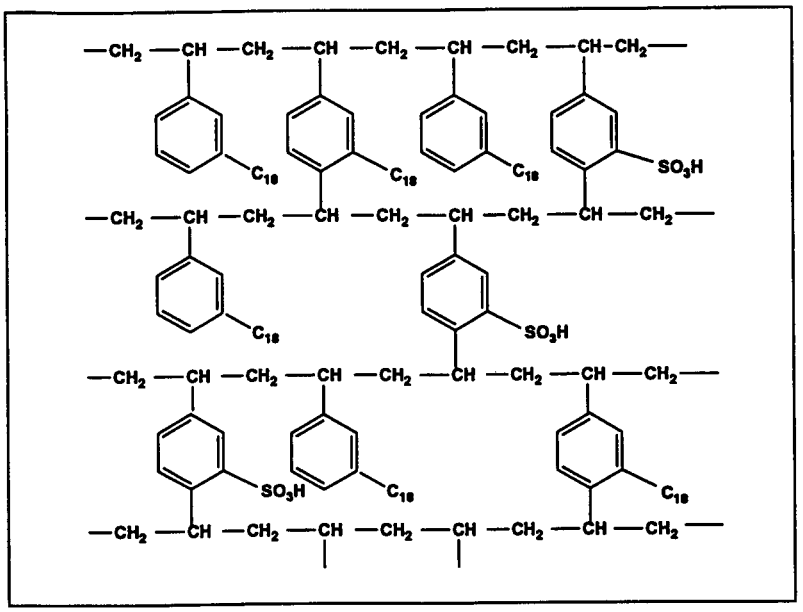
Fig. 1. Structure of the *s*-triazine suite of herbicides.

The capacity of silica-based C_{18} resin for isolation of deisopropylatrazine from pure water was compared to the capacity of two other resins —a mixed-mode resin (MP-3) and a polymeric reversed-phase resin (MP-1). Fig. 2 shows the structure of the two polymeric resins. MP-3 comprises a styrene–divinylbenzene framework onto which both C_{18} and sulfonic acid groups are bonded. MP-1 comprises a styrene–divinylbenzene framework with bonded C_{18} groups only. The C_{18} reversed-phase covering is comparable for both MP-1 and MP-3.

Fig. 3A shows the breakthrough curves for deisopropylatrazine in pure water on the three resins. Deisopropylatrazine was retained the least on MP-1, with over 10% breakthrough occurring after 10 ml of water had passed through the resin, and 100% breakthrough between 30 and 40 ml. On the silica-based C_{18} resin, 10% breakthrough of deisopropylatrazine occurred after 30 ml, and 100% breakthrough was reached after between 60 and 70 ml of water had been passed through the resin. The MP-3 resin had the largest retention for deisopropylatrazine, with 10% breakthrough still not reached after 1.8 l of water had passed through the resin. The large retention on the MP-3 resin suggests that a polar or hydrogen-bonding interaction is occurring between analyte and sulfonic acid groups, as well as Van der Waals interactions. The pK_b of the triazine compounds is small (< 2.0), [19] and at pH 7.0, the triazine compounds remain neutral analytes, which rules out the possibility of any cation-exchange interactions. It is hypothesized, therefore, that polar interactions in the form of hydrogen bonding occur between the primary amine moiety of the metabolite and the hydrogen of the sulfonic acid moiety.

The breakthrough of deisopropylatrazine on C_{18} and MP-3 was repeated out of tap water, which had

MP-3



MP-1

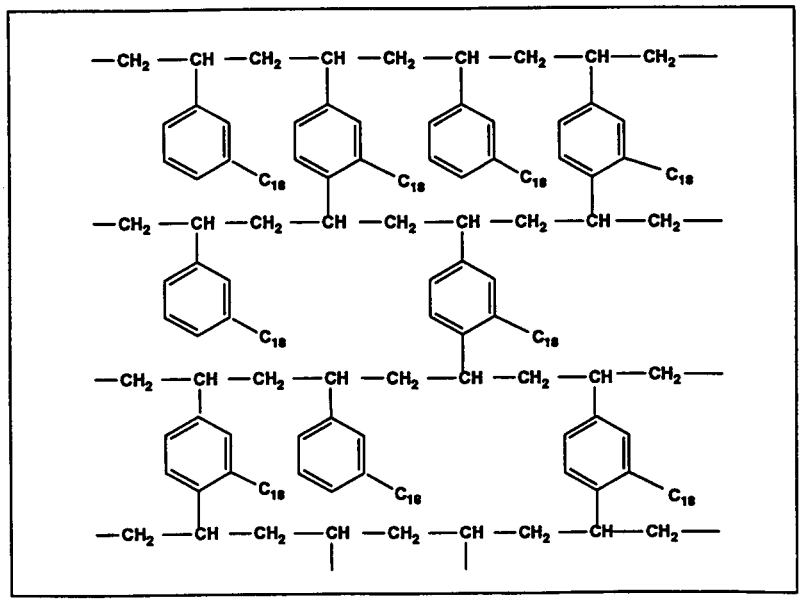


Fig. 2. Structure of copolymerized resin, MP-3, which comprises a styrene–divinylbenzene framework onto which both C_{18} and sulfonic acid groups (0.8 mequiv./g) are bonded, and MP-1, which comprises a styrene–divinylbenzene framework onto which only C_{18} groups are bonded.

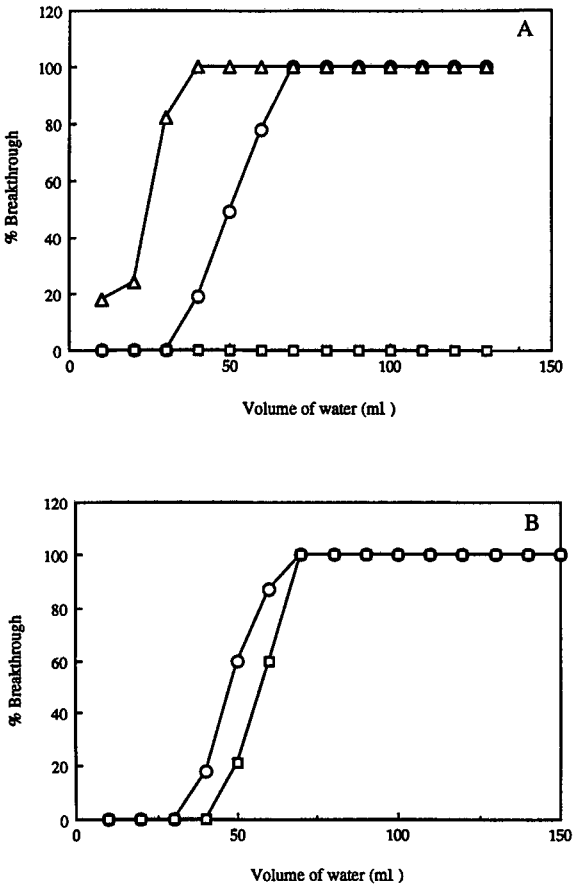


Fig. 3. (A) Breakthrough curves for deisopropylatrazine in pure water on (○) silica-based C_{18} resin, (△) polymeric reversed-phase resin (MP-1) and (□) copolymerized mixed-mode resin (MP-3). (B) Breakthrough curves for deisopropylatrazine in tap water (conductivity 200 μs) on (○) silica-based C_{18} resin and (□) copolymerized mixed-mode resin (MP-3).

a conductivity of 200 μs (Fig. 3B). The breakthrough of deisopropylatrazine on the C_{18} resin was comparable to the breakthrough out of pure water and was unaffected by the inorganic ions in solution. On the MP-3 resin, however, over 10% breakthrough occurred after only 50 ml of tap water had passed through the resin, and 100% breakthrough occurred after 70 ml of water. This indicates that hydrogen-bonding interactions were cancelled by displacement of the sulfonic acid hydrogen by the positive cations in solution. This is strong evidence that hydrogen bonding is the mechanism enhancing retention of the triazines on MP-3, because as soon as the resin is not in hydrogen-saturated form, breakthrough of deisopropylatrazine occurs rapidly.

Further evidence for a hydrogen-bonding mechanism of retention for the triazine suite was obtained from the isolation characteristics of the herbicides and metabolites on the MP-1 and MP-3 resins from organic solvents of different polarities. The retention of all compounds from methanol, ethyl acetate, acetonitrile and hexane onto the MP-1 resin was zero (all analytes remained in the organic solvent), which indicates weak Van der Waals interaction of the analytes with the non-polar styrene–divinylbenzene matrix and the C_{18} chains in comparison with that between analytes and the organic solvent. Fig. 4 shows recoveries of the triazine suite isolated from the same solvents on the MP-3 resin. From the polar solvents methanol, ethyl acetate and acetonitrile, there was smaller retention of polar triazine metabolites deisopropylatrazine and deethylatrazine, and larger retention of the remaining parent herbicides (Fig. 4). From the non-polar solvent hexane retention of the polar metabolites was 65–75%, and retention of parent triazine was 100%. Due to the presumed greater solubility of triazines in more polar solvents, such as methanol, ethanol, acetonitrile, and ethyl acetate, the retention of analytes by MP-3 resin through hydrogen-bonding interactions is decreased. As the polarity of the solvent decreases (hexane), triazine interactions with the MP-3 resin are enhanced. It appears that the solubilities of all triazines become more comparable in the solvent as the solvent polarity decreases, and the difference in retention of members of the triazine suite diminishes.

Sorption isotherms were conducted for four members of the triazine suite, deisopropylatrazine, deethylatrazine, atrazine and ametryn, from distilled water on MP-3 and MP-1 resins, at small concentrations relative to the solubility of the analytes. Linear sorption isotherms for all analytes on the MP-3 and MP-1 resins were obtained. Chiou [20] has emphasized that when the concentration of the solute is small relative to the solutes solubility, a linear sorption isotherm will result, as stoichiometric interactions are incomplete. At larger solute concentrations, such interactions are complete, and the isotherm will become non-linear. The isotherm for

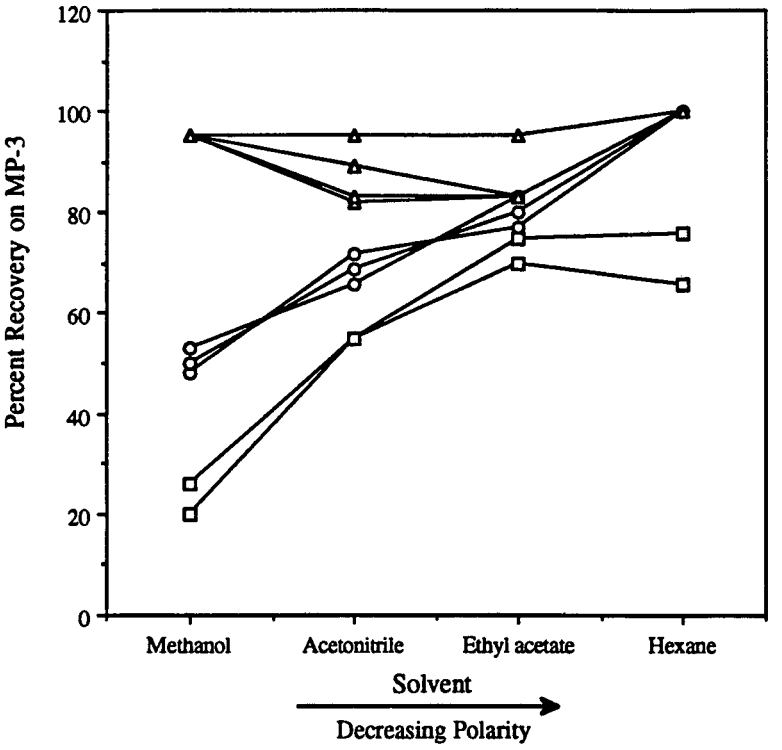


Fig. 4. Recovery of triazine suite on MP-3 mixed-mode resin isolated from polar and non-polar organic solvents. △ = Prometon, ametryn, prometrym terbutryn; ○ = simazine, atrazine, propazine; □ = deisopropylatrazine, deethylatrazine.

the MP-3 resin may become non-linear at larger solute concentrations, and this would support a secondary interaction taking place to explain the enhanced isolation of the triazine compounds.

The slope of the plot of analyte sorbed onto the solid phase (ng/g) *versus* the concentration of analyte in the water phase (ng/ml) is defined as the partition coefficient (K_d). Table I lists the K_d values for each analyte on MP-1 and MP-3 resins. Because the MP-1 and MP-3 resins differ only in structure by the presence of sulfonic acid groups, the difference in the log K_d for MP-1 and MP-3 resins (Δlog K_d) for the same analyte can be attributed to the enhanced interactions (hydrogen bonding) with the sulfonic acid groups. The Δlog K_d value was approximately 1.9 for both triazine metabolites, each of which contain a primary amine moiety. From the expression [$\Delta G = 2.303RT(\Delta \log K_d)$], the free energy of adsorption (ΔG) associated with the enhanced interactions is 2.59 kcal/mol. This is in the energy range for hydrogen bonding. The Δlog K_d value for the parent herbicides, atrazine and ametryn, which both contain secondary amine moieties, was approximately 1.3, which equates to an energy of adsorption of 1.77 kcal/mol. These Δlog K_d values and small energies of adsorption indicate that the enhanced interactions could be due to hydrogen-bonding interactions on the MP-3 resin, and that

TABLE I

PARTITION COEFFICIENTS (K_d VALUES) FOR DEISOPROPYLATRAZINE, DEETHYLATRAZINE, ATRAZINE AND AMETRYN ON MP-1 AND MP-3 POLYMERIC RESINS

Compound	K_d (ml/g)	
	MP-1	MP-3
Deisopropylatrazine	400	58 600
Deethylatrazine	1600	112 500
Atrazine	40 000	625 000
Ametryn	184 600	4 000 000

TABLE II

PERCENT RECOVERY OF THE TRIAZINE SUITE (± 5%) FROM PURE WATER FOLLOWED BY A METHANOL WASH ON COPOLYMERIZED AND BLENDED MIXED-MODE RESINS

Compound	Recovery (%)	
	Co-polymerized mixed-mode	Blended mixed-mode
Deisopropylatrazine	95	61
Deethylatrazine	95	65
Ametryn	95	95
Atrazine	95	65
Prometon	95	95
Prometryn	95	95
Propazine	95	65
Simazine	95	65
Terbutryn	95	95

these interactions are stronger between primary amine groups and the sulfonic acid groups than between the secondary amines.

A comparison was made of the recovery of the triazine compounds on two types of commercially available mixed-mode resins —the co-polymerized mixed-mode resin (MP-3) and a blended mixed-mode resin, where a mixture of reversed-phase resin and cation-exchange resin are combined in one SPE cartridge. Table II shows recovery of the triazine suite from pure water followed by a 6-ml methanol-wash step prior to elution with ethyl acetate containing 2% ammonium hydroxide. Recovery of the triazine compounds from pure water was comparable for the two types of resin, with recoveries of 95% for all triazine herbicides and metabolites. When a methanol-wash step was added following isolation, recoveries of polar metabolites and the parent herbicides, atrazine, propazine, and simazine decreased to 65% on the blended mixed-mode resin but remained unchanged on the copolymerized mixed-mode resin. The recovery of the more nonpolar parent triazine herbicides, ametryn, prometon, prometryn and terbutryn remained at 95%. This result indicates that the proximity of the cation-exchange moiety to the C_{18} phase is important in ensuring full recovery of more polar analytes when using organic solvents as part of the procedure. Because cation-exchange and C_{18} reversed-phase moieties are separated by the particle size of the silica in blended mixed-mode resins, the analyte is retained by either reversed-phase or secondary interactions, and is not dually held by both mechanism. During a methanol-wash step, a polar triazine retained by Van der Waals interactions only is easily eluted. Only the analyte retained by hydrogen-bonding interactions will remain. The less-polar triazine compounds also will be eluted with methanol from a relatively non-polar organic phase, as evidenced by zero retention of any triazine herbicides or metabolites out of organic solvent on the MP-1 resin. However, the more non-polar compounds can partition more readily out of the polar solvent and back onto a hydrogen-bonding site than polar compounds, as seen by their consistently large recoveries out of organic solvents onto MP-3. The close proximity of the two moieties on the copolymerized mixed-mode resin is important, therefore, to hold the more polar compounds by a dual mechanism at all times. Van der Waals interactions can then be disrupted with a methanol wash, but the triazine is retained by the secondary hydrogen-bonding interactions with the sulfonic acid group.

Mixed-mode isolation of benzoylecgonine

To design an efficient method for solid-phase extraction of benzoylecgonine, first it is necessary to examine the structure of the drug and its basicity. Fig. 5 shows the structure of benzoylecgonine. It is an amphoteric compound with both a carboxyl group and a tertiary amine. Thus, in a SPE procedure, the drug may be sorbed most efficiently at a neutral to slightly acidic pH by using a non-specific partition interaction, such as with C_8 resin. At an acid pH, benzoylecgonine will be a cation with protonation of the tertiary amine. This side of the drug's structure can be exploited by cation exchange.

First, the SPE procedure isolates the benzoylecgonine with passage of the urine sample through the

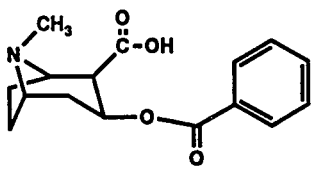


Fig. 5. Structure of benzoylecgonine.

TABLE III

RECOVERY OF BENZOYLECGONINE FROM VARIOUS MIXED-MODE RESINS

Resin	Recovery of benzoylecgonine (%)
Blended SPE 1 resin	60
Blended SPE 2 resin	70
Mixed-mode MP-3 resin (copolymerized)	95
C_{18} resin followed by cation-exchange resin	90

cartridge at pH 6.0. At this pH, the drug is ionized at the carboxyl group but should adsorb by Van der Waals interactions to the C_8 resin. Many urine interferants also will sorb at this pH. To wash the resin of these interferants, first water is used to remove excess salts and extremely soluble organic compounds. Next, acid is used to regenerate the cation-exchange resin, which has lost some of its hydrogen saturation by introduction of the urine sample. Next, an organic solvent, such as methanol, is required. However, if methanol is used, it will elute the benzoylecgonine at the same time. To remove the interferants without loss of the drug, the basic side of the molecule can be exploited by use of acid. By passing acid through the column, not only will the cation-exchange capacity be regenerated but also the carboxyl group will be protonated, and the basic amine will become cationic. Thus, a SPE cartridge that contains both a hydrophobic group and a strong cation-exchange group should work well on this compound and indeed on any basic drug.

This procedure was tested on four different mixed-mode resins (Table III). Two blended mixed-mode resins from different manufacturers, consisting of C_8 and strong cation-exchange resins in their hydrogen-saturated form, were compared with a copolymerized mixed-mode resin (MP-3) and a combination of C_{18} resin followed by a strong cation-exchange resin. The blended resins were commerically available. The recovery of benzoylecgonine varied from 60 to 70% of the applied amount for the blended resins. This decreased recovery probably is caused by the fact that the benzoylecgonine can desorb from the C_8 resin and re-adsorb by cation exchange on an adjacent resin bead.

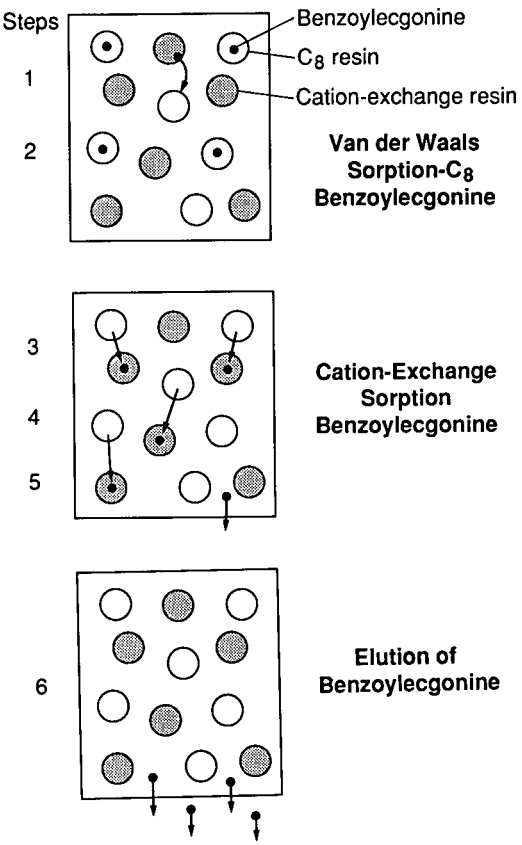


Fig. 6. Procedure and mechanism of isolation of benzoylecgonine on blended mixed-mode resin. White circles: C_8; grey circles: AR-SO_3H. Steps: 1 = condition column; 2 = apply sample with benzoylecgonine; 3 = wash with water; 4 = acid wash (proton lock); 5 = methanol wash; 6 = elute with methylene chloride–isopropanol (80:18) with 2% ammonium hydroxide.

For example, the method depicted in Fig. 6 shows that the blended resin contains both C_8 and cation-exchange beads. During the initial phase of sorption, the C_8 beads sorb the benzoylecgonine via Van der Waals interactions. During the water wash, the urine interferants are washed out, but the benzoylecgonine remains sorbed by the C_8 resin. The methanol-wash step, however, elutes the benzoylecgonine from the C_8 resin. The data in Table II indicate this result by the 90% recovery of benzoylecgonine when the methanol eluate of the C_{18} resin is passed through the cation-exchange resin. However, because an acid-wash step precedes the methanol rinse, the carboxyl group of the benzoylecgo-

nine is protonated, and the tertiary amine takes on a positive charge. This cation then is retained on the cation-exchange resin that is blended in the SPE cartridge during the methanol wash step. Thus, the molecules must desorb from the reversed-phase bead and move to a cation-exchange bead for retention. Consequently, in the blended resin some of the molecules are washed from the cartridge before the cation exchange occurs. Because cation exchange often is a slow process the flow-rate may easily affect recovery of the benzoylecgonine (Table III, recovery 60–70%).

When the resin contains both the reversed-phase and ion-exchange groups in close proximity, as on the co-polymerized MP-3, then the recovery is nearly total (95%). This may be explained easily by the desorption of the benzoylecgonine from the hydrophobic matrix and the immediate sorption by cation exchange without transport to the next bead. Thus, recovery is high in spite of the large wash volume of 9 ml of methanol.This mechanism of sorption, which is activated by an acid wash with subsequent sorption by cation exchange, is called a "proton-locking" step. This procedure is quite important for all of the classes of basic drugs that contain nitrogen that are easily protonated by acid.

The methanol-wash step on the mixed-mode resins was quite effective at removing nonvolatile, colored urinary metabolites. The eluates from C_8 resins were more colored than eluates from the mixed-mode resins because they were not washed with methanol. Injection of non-volatile urinary acids onto the gas chromatograph will shorten the life of the column due to activation, reduce sensitivity, and cause peak broadening. Furthermore, cleaner chromatograms were obtained with the mixed-mode eluates compared to the C_8 eluates. Thus, the application of mixed-mode resins to basic drugs in urine provides an efficient clean-up procedure.

CONCLUSIONS

The retention of both polar *s*-triazine metabolites and non-polar parent herbicides on copolymerized mixed-mode resin MP-3 is achieved by a combination of Van der Waals and hydrogen-bonding interactions. The dual-retention mechanism is demonstrated by retention of all triazine compounds from both polar and nonpolar organic solvents. Hydrogen-bonding interactions were completely disrupted only by adding 2% ammonium hydroxide to the eluting solvent; therefore, organic solvents can be used to selectively elute any interferents from the resin held by Van der Waals interactions only. Adsorption isotherms for the triazine compounds were linear on the MP-1 and MP-3 resins, with a difference in free energy of sorption between the two resins of 1.77 kcal/mol for parent triazine herbicides and 2.59 kcal/mol for the polar triazine metabolites. This is in the range of hydrogen-bonding interactions.

The basic drug, benzoylecgonine, was retained from urine on mixed-mode resin by a combination of Van der Waals and cation-exchange interactions. The basic drug was applied to the resin in neutral form and protonated "on resin" by an acid-wash step, which also re-protonated the cation-exchange groups. During a methanol-wash step, Van der Waals interactions were disrupted, but the drug was held by cation exchange while neutral interferences were washed off. The drug was eluted with organic solvent containing 2% ammonium hydroxide.

A comparison of retention efficiency of copolymerized and blended mixed-mode resins showed smaller recoveries for polar triazine compounds and benzoylecgonine on blended resin and larger recoveries on the copolymerized resin. This result indicates that the closeness of reversed-phase and sulfonic acid moieties is important. In blended resins, these groups are separated physically by the particle size of the silica. Mixed-mode resins are an innovative contribution to solid-phase sorbents for the enhanced isolation of polar analytes with non-polar analytes from complex matrices.

ACKNOWLEDGEMENTS

The authors wish to thank Ragina Patel (Bio-Rad, Hercules, CA, USA) and David Hometchko (Interaction Chromatography, San Jose, CA, USA) for their help and assistance in supplying mixed-mode resins.

REFERENCES

1 P. D. MacDonald (Editor), *Waters Sep-Pak Cartridge Applications Biobliography*, Waters, Milford, MA, 5th ed., 1991.
2 *Applications Bibliography Sample Preparation Products*, Varian, Harbor City, CA, 1992.

3 R. M. Patel, J. J. Jagodzinski, J. R. Benson and D. Hometchko, *LC · GC*, 8 (1990) 874.
4 R. M. Patel, J. R. Benson, D. Hometchko and G. Marshall, *Am. Lab.*, Feb (1990) 92.
5 V. Dixit and V. M. Dixit, *Am. Lab.*, May/June (1990) 47.
6 V. Dixit and V. M. Dixit, *Indian J. Chem.*, 30B (1991) 164.
7 H. Engelhart and M. Jungheim, *Chromatographia*, 29 (1990) 59.
8 J. Kohler, D. B. Chase, R. D. Farlee, A. J. Vega and J. J. Kirkland., *J. Chromatogr.*, 352 (1986) 275.
9 Z. Matus and R. Omacht, *Chromatographia*, 30 (516) (1990) 318.
10 S. G. Weber and W. G. Tramposch, *Anal. Chem.*, 55 (1983) 1771.
11 V. Marko and K. Radova, *J. Liq. Chromatogr.*, 14 (1991) 1671.
12 V. Marko, L. Soltes and I. Novak, *J. Pharm. Biomed. Anal.*, 8 (1990) 297.
13 R. J. Ruane and I. D. Wilson, *J. Pharm. Biomed. Anal.*, 5 (1987) 723.
14 M. Moors and D. L. Massart, *J. Pharm. Biomed. Anal.*, 9 (1991) 129.
15 A. Di Corcia and R. Samperi, *Anal. Chem.*, 62 (1990) 1490.
16 M. Battista, A. Di Corcia and M. Marchetti, *Anal. Chem.*, 61 (1989) 935.
17 E. M. Thurman, M. Meyer, M. Pomes, C. A. Perry and A. P. Schwab, *Anal. Chem.*, 62 (1990) 2043.
18 M. S. Mills and E. M. Thurman, *Anal. Chem.*, 64 (1992) 1985.
19 V. Pacakova, K. Stulik and M. Prihoda, *J. Chromatogr.*, 442 (1988) 147.
20 C. T. Chiou, in B. L. Sawhney and K. Brown (Editors), *Reactions and Movement of Organic Chemicals in Soils*, Soil Science Society of America, Madison, WI, 1989, p. 1.

Journal of Chromatography, 629 (1993) 23–34
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 697

Disruption and fractionation of biological materials by matrix solid-phase dispersion

Steven A. Barker, Austin R. Long* and Murray E. Hines II**

Laboratory for Residue Studies, Department of Physiology, Pharmacology and Toxicology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803 (USA)

ABSTRACT

The isolation of drug residues, environmental contaminants or naturally occurring component molecules from biological materials is often a complex undertaking. We report here the development and application of a simple approach to the disruption of biological samples that also allows for the rapid fractionation and isolation of the sample's natural components or incurred residues. This process, called matrix solid-phase dispersion (MSPD), combines the use of mechanical forces generated from the grinding of samples with irregular shaped particles (silica or polymer based solid supports) with the lipid solubilizing capacity of a support-bound polymer (octadecylsilyl or others) to produce a sample/column material from which dispersed sample matrix components can be selectively isolated. The factors governing this process and examples of its various applications are presented.

INTRODUCTION

Methods for the isolation of target molecules from biological matrices, such as tissues, often begin with a process designed to disrupt the general architecture of the sample. By using a mechanical blender or by grinding with abrasives, such as sand, the sample is reduced to fragments of structural components or clusters of cells. A degree of cell lysis occurs and can be enhanced by sonication, extrusion or the addition of chemical agents. To assist in lysis, one may treat the membrane fragments and subcellular structures with surfactants, which tend to completely disrupt and solubilize the component molecules. Such a step is necessary in the isolation of integral membrane proteins or molecular complexes.

In the case of animal cells, relatively mild procedures accomplish cell lysis. However, plants, bacteria and fungi possess cell walls and often require more physically and chemically dynamic procedures to obtain complete cellular disruption. This may involve the weakening of the cell wall by enzymes, extended sonication at maximum intensity, and the use of high concentrations of detergents. Although these processes accomplish cellular disruption their physical and chemical harshness can complicate the procedures for the isolation of the target molecules or lead to their destruction and the generation of sample artifacts. These factors are of concern regardless of the methodology employed.

The fractionation of the lysates involves methodology to isolate a specific type of target compound (*i.e.*, lipid, carbohydrate, protein, peptide, drug, metabolite, pollutant, etc.), often to the exclusion of all others. Such isolation techniques may involve centrifugation, counter-current extractions, pH adjustments, various forms of chromatography or combinations of these and other technologies. While accomplishing the desired task this overall

* Present address: Animal Drug Research Center, PHS/HHS/FDA, Bldg. 20, Room 1121, Denver Federal Center, Lakewood, CO 80225-0087, USA.

** Present address: Department of Comparative Pathology, Box 016960 R-46, School of Medicine, University of Miami, Miami, FL 333101, USA.

Correspondence to: S. A. Barker, Lab. for Residue Studies, Department of Physiology, Pharmacology and Toxicology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803, USA.

approach can be extremely laborious and time and materials intensive. Furthermore, the high degree of sample manipulation often leads to poor recoveries and, thus, an inefficient process.

For the last several years our laboratory has been involved in developing procedures for the isolation of drug residues from tissues using a process called matrix solid-phase dispersion (MSPD) [1]. This process involves the grinding of biological samples with silica solid supports (40 μm particle size) to which lipid solubilizing polymers (octadecylsilyl or others) are chemically bound. We present here a summary of data obtained to date in examining the application of MSPD to the isolation of drug and pollutant residues from a variety of biological matrices. We also present data illustrating its application to the disruption and fractionation of muscle tissue and the bacteria *Mycobacterium paratuberculosis* and *Escherichia coli*. The results of these studies indicate that MSPD is a generic method for the disruption, lysis and fractionation of biological matrices that may possess numerous advantages when compared to classical approaches.

EXPERIMENTAL

Materials

Liquid chromatographic grade solvents from commercial sources were used without further purification. Water for reagents and HPLC mobile phases was triple distilled and passed through a Modulab Polisher I water purification system. Bulk octadecylsilane (C_{18}) derivatized silica (40 μm, 18% carbon load, endcapped from Analytichem, Harbor City, CA, USA) was washed with hexane, dichloromethane (DCM) and methanol and was air dried prior to use. Syringe barrels of 10 ml were thoroughly washed with hot, soapy water, rinsed with distilled water and methanol and air dried for preparation of MSPD columns. Filter paper discs (1.5 cm diameter, Whatman No. 1) were used as column frits to retain the column packing. Standards for the various substances tested were obtained from commercial sources and were >98% purity.

Generic procedures

By adding 0.5 g of sample (milk, fat, liver, kidney, muscle, cultered cell pellet, etc.) to C_{18} derivatized silica or some other appropiate lipophilic polymer-derivatized silica or polymer solid support column packing (2.0 g) in a glass or agate mortar and gently grinding the material for 30 s with a pestle, a nearly homogeneous blend of sample components "dissolved" or dispersed on the solid phase packing material can be obtained. It may be necessary to scrape the sides of the mortar and pestle and repeat this process of blending when dealing with particularly "wet" samples or if homogeneity is not evident. The blend is then transferred with a funnel to a syringe barrel column (10 ml syringe barrel) plugged with a filter paper disc (Whatman No. 1, 1.5 cm). The column head is covered with a second disc and the contents are compressed by a plunger to a volume of 4.5 ml. The column may then be eluted with a single solvent or a series of solvents in order to elute a specific compound, a class of compounds or to perform a total fractionation of the sample matrix. Further purification or the use of co-columns may be required depending on the intended use of the extract and the nature of the compounds being isolated. Elution may be performed by gravity flow, use of a vacuum manifold or centrifugation.

Isolation of drug residues

This generic method has been applied to the isolation of some thirty different compounds representing several major drug classes from a variety of tissue and sample matrices and are summarized in Table I.

Isolation of pesticides and other environmental pollutants

Polyaromatic hydrocarbons in catfish muscle [19]. Following the procedures given above the MSPD column was eluted with 8.0 ml of acetonitrile (ACN). The extract was evaporated to dryness, reconstituted in 1.0 ml of ACN, filtered and assayed by HPLC with UV and florescence detection. The method extracted 14 different fortified polyaromatic hydrocarbons (PAHs) over a concentration range of 100–2000 ng/g with recoveries ranging from 73–112%.

Pesticides in bovine fat [20] and catfish muscle [21]. Nine chlorinated pesticides were isolated from bovine fat or catfish muscle by elution of the MSPD column with 8.0 ml of ACN through a co-column of Florisil (2.0 g) with a recovery ranging from 62–114% for the concentrations examined.

TABLE I

A LISTING OF COMPOUND CLASSES FOR WHICH MSPD EXTRACTION METHODOLOGY HAS BEEN ESTABLISHED

Compound	Matrix (ref.)	Recovery ($n \geqslant 20$)	MSPD wash and eluting solvent[a]
Aminoglycosides			
Neomycin	Bovine kidney [2]	88.6 ± 4.6	Cyanopropyl; wash; hexane, ethyl acetate, MeOH, water; elute 0.1 *M* HCl
Avermectin			
Ivermectin	Bovine liver [3]	74.9 ± 7.3	Wash; hexane, DCM–ethyl acetate (3:1), ACN; elute MeOH
Benzimidazoles			
Albendazole	Milk [14]	81.1 ± 6.8	Wash; hexane; elute, DCM–ethyl acetate (1:2)
	Bovine muscle [1]	73.9 ± 8.0	Wash; hexane, benzene; elute, ethyl acetate
	Bovine liver [13]	72.4 ± 2.6	Wash; hexane; elute, ACN
	Swine muscle [12]	93.0 ± 6.2	Wash; hexane; elute, ACN
Fenbendazole (FBZ)	Milk [14]	69.7 ± 8.9	
	Bovine muscle [1]	74.0 ± 11.8	
	Bovine liver [13]	62.0 ± 5.3	
	Swine muscle [12]	98.0 ± 5.3	
FBZ-OH	Milk [14]	94.4 ± 5.1	
	Bovine muscle [1]	68.4 ± 10.5	
FBZ-SO_2	Milk [14]	100 ± 4.1	
	Bovine muscle [1]	85.7 ± 15.0	
Mebendazole	Milk [14]	101 ± 4.1	
	Bovine muscle [1]	63 ± 4.2	
	Bovine liver [13]	93.0 ± 5.7	
	Swine muscle [12]	85.2 ± 3.2	
Oxfendazole	Milk [14]	107 ± 2.3	
	Bovine muscle [1]	82.9 ± 9.5	
	Bovine liver [13]	86.8 ± 10.8	
	Swine muscle [12]	92.2 ± 7.8	
Thiabendazole	Milk [14]	88.7 ± 5.8	
	Bovine muscle [1]	63.8 ± 9.6	
	Bovine liver [13]	78.5 ± 1.0	
	Swine muscle [12]	85.5 ± 6.8	
β-Lactams			
Penicillin	Bovine muscle [1]	86.3 ± 6.1	Wash; hexane, benzene, ethyl acetate; elute, methanol
Ampicillin	Bovine muscle [1]	59.8 ± 9.8	
Cephalosporins			
Cephapirin	Bovine muscle [1]	72.4 ± 26.5	Wash; hexane, benzene, ethyl acetate; elute, MeOH
Chloramphenicol	Milk [4]	68.7 ± 8.3	Wash; hexane, benzene; elute, ethyl acetate
Chlorsulon	Milk [5]	99.8 ± 5.3	Diethyl ether
Furazolidone	Swine muscle [7]	89.5 ± 8.1	Wash; hexane; elute, DCM
	Chicken muscle [8]	89	Wash; hexane; elute, DCM
	Milk [6]	81.7 ± 8.0	Wash; hexane; elute, DCM
Nicarbazin	Chicken liver [9]	87.8 ± 1.9	Wash; hexane; elute, ACN
	Chicken muscle [9]	84.4 ± 7.9	
Sulfonamides (S = sulfa)			
S-diazine	Swine muscle [17]	95.1 ± 15.1	Wash; hexane; elute, DCM
	Milk [15]	81.2 ± 4.8	Wash; hexane; elute, DCM
	Infant formula [16]	99.6 ± 5.3	Wash; hexane; elute, DCM

(Continued on p. 26)

TABLE I *(continued)*

Compound	Matrix (ref.)	Recovery ($n \geq 20$)	MSPD wash and eluting solvent[a]
S-dimethoxine	Swine muscle [17]	95.8 ± 12.4	
	Milk [15]	89.6 ± 8.1	
	Infant formula [16]	103 ± 9.2	
	Catfish muscle [10]	101.1 ± 4.2	Wash; hexane; elute, DCM
S-merazine	Swine muscle [17]	78.1 ± 9.1	
	Milk [15]	82.0 ± 4.5	
	Infant formula [16]	92.7 ± 8.8	
S-methazine	Swine muscle [17]	84.7 ± 8.2	
	Milk [15]	92.7 ± 5.6	
	Infant formula [16]	99.1 ± 8.8	
S-methoxazole	Swine muscle [17]	95.7 ± 14.8	
	Milk [15]	89.4 ± 8.3	
	Infant formula [16]	112 ± 8.2	
S-anilamide	Swine muscle [17]	70.4 ± 12.7	
	Milk [15]	73.1 ± 7.3	
S-thiazole	Swine muscle [17]	80.3 ± 11.1	
	Milk [15]	93.7 ± 2.7	
	Infant formula [16]	75.9 ± 11.1	
Sulfisoxazole	Swine muscle [17]	92.8 ± 11.8	
	Milk [21]	88.6 ± 11.2	
	Infant formula [22]	93.1 ± 9.7	
Tetracyclines			
Chlortetracycline	Milk [18]	77.2 ± 11.3	Wash; hexane; elute, ACN–ethyl acetate (3:1)
Oxytetracycline	Milk [18]	93.3 ± 3.4	
	Fish [11]	80.9 ± 6.6	Wash, hexane; elute, ACN–MeOH (1:1)
Tetracycline	Milk [18]	63.5 ± 19.6	

[a] Washing sequences are the same for compounds of the same class and for the same reference number.

The resulting eluate was assayed directly by GC–electron-capture detection.

Pesticides in oysters [22] and crawfish and lobster [23] hepatopancreas. Similarly, oyster or crustacean hepatopancreas homogenate was eluted with 8.0 ml of ACN—MeOH (9:1, v/v) through a Florisil co-column. The process isolated 14 chlorinated pesticides with recoveries greater than 60% for concentrations ranging from 62–2000 ng/g of tissue. The eluates were assayed, without further manipulation of the sample, by GC with electron-capture detection.

Clorsulfuron in milk [24]. The milk/C_{18} MSPD column was eluted with 9.0 ml of hexane. The hexane was discarded and the column was eluted with 9.0 ml of DCM. The solvent was removed by evaporation with nitrogen, reconstituted in 250 μl of DCM and analysed by GC with nitrogen/phosphorous detection.

Disruption, lysis and fractionation of bovine muscle tissue [25]

Samples were prepared as described above. Several small aliquots were removed and were sprinkled onto scanning electron microscopy (SEM) mounts coated with a thin layer of graphite paint. C_{18} Material alone served as a control. After drying overnight, the excess C_{18}/sample blend was removed by abrupt shaking. The coated mount was then shadowed with gold–palladium and viewed with a Cambridge Stereoscan Model F-150 scanning electron microscope.

The MSPD column was eluted with 8.0 ml each of the following solvents, hexane, DCM, ethyl acetate, ACN, MeOH and water, respectively. The eluates were collected in preweighed conical glass tubes and the solvents were evaporated under a stream of dry nitrogen. The tubes were then reweighed to determine mass balance. The column packing was removed and sonicated in normal sa-

line. Insoluble materials (other than the C_{18} itself) were removed and assayed. Analyses for the determination on the various classes of compounds in each of the different eluates were undertaken. (i) Protein content by the method of Lowry described in ref. 26. (ii) Total cholesterol by the Sigma colorimetric/enzymatic assay, Procedure No. 352. (iii) Triglycerides were assayed by TLC [27]. (iv) Free fatty acid content and bound fatty acid content (triglycerides, phospholipids, etc.) were determined by differential GC–MS analysis, comparing fatty acid content with and without hydrolytic methanolysis of the extract residues. GC–MS analysis of residues with and without derivatization (trimethylsilyl, TMS) afforded identification of several mono- and disaccharides as well as other individual compounds or compound classes [25].

Lysis and fractionation of bacteria [27]

Samples of pelleted *M. paratuberculosis* and *E. coli* (0.5 g) were blended with 3.0 g and 2.0 g of the C_{18} material, respectively. Aliquots were taken for SEM analysis as described above. Similarly, pelleted bacteria were blended with 40 μm underivatized silica particles, containing no octadecylsilane, for comparison.

Columns prepared from these blends were sequentially eluted with 12.0 ml each of hexane, DCM, ACN, MeOH and water into preweighed conical glass tubes. The organic solvents were removed by evaporation with nitrogen and the water extract was lyophilized. The tubes were then reweighed for determination of mass balance. Protein content in the various fractions was determined by the method of Markwell described in ref. 28. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was also performed on all fractions [29,30]. Nucleic acid content was determined by measurement of absorbance at 260 nm and the 260 nm/280 nm ratio [31] and by the presence of florescent bands on a 1.0% agarose minigel after staining with ethidium bromide [32]. Phospholipid content was determined by a direct colorimetric method [33]. Quantitation of *E. coli* lipopolysaccharide content was conducted by the method of Karkhanis described in ref. 34. As described above for muscle tissues, the various eluates were also examined by differential GC–MS analyses to determine the presence of other compounds or compound classes.

RESULTS AND DISCUSSION

A summary of results utilizing the MSPD technique for the isolation of drugs from a variety of biological matrices is given in Table I. Similarly, the results of the application of this method to the isolation of chlorinated pesticides and organophosphates from several aquatic and non-aquatic species are given in Table II. Mass balance data for *M. paratuberculosis* is shown in Table III. The identification of various classes of compounds for the bacteria are given in Table IV. Micrographs from the examination of the materials obtained by blending the silica particles with tissue and bacteria are shown in Fig. 1A–E.

The extraction methods for the various drug classes summarized in Table I provided recoveries of greater than 60% for the individual compounds, whether the method was for a given compound or for several compounds, over the range of concentrations examined. All of the procedures developed gave correlation coefficients for linearity of 0.99 or better. The limits of detection obtained for these drugs were at or below the action levels established for the various drugs by the different regulatory agencies at the time of publication. In most cases, no clean-up steps were required post-elution and concentration. The extracts, analyzed by HPLC or GC, were relatively free of contaminating co-extractants. In the LC analyses the drugs were assayed using simple isocratic solvent systems with relatively short run times (6–25 min), even when the analysis was conducted for several compounds simultaneously.

Similarly, the methodlogy developed for the chlorinated pesticides and organophosphates, and summarized in Table II, provided more than adequate limits of detection and excellent recoveries and linearities over the ranges of concentrations tested in the various matrices. The cleanliness of the extracts also greatly enhanced the analyses and reduced the need for any post-elution clean-up. Essentially the samples are assayed directly after elution, reducing the degree of sample manipulation required and the time necessary to obtain results. As with many of the drug methods, the MSPD approach required a minimum of 8.0 ml of solvent and could be performed within 30 min, ready for analysis.

There are several points to be made concerning

TABLE II

THE PERCENT RECOVERIES OF VARIOUS PESTICIDES FROM DIFFERENT MATRICES

Samples were extracted by MSPD and assayed as described in Experimental. Recovery values are the mean for the range of concentrations examined ± S.D.

Pesticides	Matrix					
	Catfish muscle [21]	Bovine fat [20]	Oyster [22]	Crawfish [23]	Milk [24]	Bovine muscle [1]
Lindane	82 ± 5	85 ± 3	78 ± 7	86 ± 7		
Heptachlor	84 ± 9	86 ± 5	73 ± 8	86 ± 10		
Aldrin	94 ± 12	92 ± 13	67 ± 10	80 ± 12		
Heptachlor epoxide	93 ± 12	86 ± 6	82 ± 10	74 ± 9		
p,p'-DDE	91 ± 6	94 ± 6	81 ± 11	94 ± 13		
Dieldrin	91 ± 2	95 ± 3	73 ± 11	90 ± 11		
Endrin	93 ± 7	97 ± 3	74 ± 9	79 ± 2		
p,p'-TDE	97 ± 4	97 ± 5				
p,p'-DDT	97 ± 5	102 ± 5	69 ± 12	96 ± 13		
a-BHC			77 ± 8	83 ± 6		
b-BHC			80 ± 16	81 ± 4		
4,4'-DDD			81 ± 14	90 ± 9		
Endosulfan-SO_4			74 ± 8	93 ± 12		
Methoxychlor			65 ± 8	95 ± 12		
Endrin aldehyde			70 ± 7	54 ± 7		
Clorsulfuron					92 ± 11	
Fenthion						86 ± 8
Coumaphos						77 ± 8
Famfur						82 ± 9
Crufomate						94 ± 6

these results as they relate to the MSPD process. The compounds extracted represent a diversity of molecular structure and polarity characteristics. Nevertheless, an essentially generic methodology afforded high recoveries, with a degree of specificity, of all of these various compounds from several different matrices, even from the same sample. The mechanisms involved in MSPD appear to encompass sample homogenization and cellular disruption, exhaustive extraction, fractionation and purification in a single process. The method involves the dispersal of a sample over a theoretical surface area of 1000 m^2 (500 m^2/g C_{18} solid support) in a thin film (100 Å), utilizing the shear forces of the particles and the blending or grinding action employed to disrupt the sample architecture while the polymer (C_{18} or others) serves to dissolve or disperse the sample components on the basis of hydrophobic–hydrophilic interactions. In this manner the polymer bound to the solid support may literally disrupt and unfold cell membrane or micellar lipids. The disruption process can be envisioned as incorporating the use of shear forces from the particles with tissue solubilization using detergents, two classical approaches to tissue and cellular disruption. However, in MSPD the "detergent" is bound to the particles, eliminating the need to subsequently remove the detergent before the final analysis, and provides a unique column support material for subsequent isolation of the dispersed compounds.

TABLE III

THE PERCENT RECOVERIES (± S.D.) AND MASS BALANCE FOR THE DISRUPTION AND FRACTIONATION OF *M. PARATUBERCULOSIS* [27]

Data are calculated based on the dry weight of the sample (0.5 g wet weight).

Solvent	% Recovery *M. paratuberculosis*
Hexane	14.7 ± 4.7
DCM	40.6 ± 3.3
ACN	16.6 ± 6.1
MeOH	17.2 ± 2.8
Water	11.2 ± 0.4
Remaining on column	6.8 ± 0.5
Total accounted for	100 ± 4.7

TABLE IV

THE MAJOR COMPOUNDS OR CLASS OF COMPOUNDS IDENTIFIED IN THE VARIOUS ELUATES OBTAINED FROM THE DISRUPTION AND FRACTIONATION OF THE BACTERIA *M. PARATUBERCULOSIS* AND *E. COLI* BY MSPD [27]

Solvent	Major compounds identified	
	M. paratuberculosis	*E. coli*
Hexane	Neutral lipids	Neutral lipids
DCM	Phospholipids	Neutral lipids
		Short chain fatty acids
		Indoles
		Quinolines
ACN	Short chain fatty acids	Pyrimidine
	Sterols	Indoles
		Aromatic acids
		Sterols
MeOH	Phospholipids	Phospholipids
	Amino acids	Amino acids
	Inositols	Purines
	Mono-, disaccharides	Pyrimidines
	Citric acid	Mono-, disaccharides
Water	Nuleotides/nucleotides	Nucleotides/nucleotides
		Lipopolysaccharides
	Protein: Water > DCM > MeOH > ACN	Protein; Water ≫ MeOH > ACN

By transferring the material to a column and performing a solvent elution one obtains a distribution of the compounds as well as other sample components that is dependent on (i) interactions with the bound polymer phase and solid support, (ii) interactions with the dispersed sample matrix components, (iii) molecular size and (iv) interactions with the eluting solvent(s). Combinations of these factors for individual components are also certain to be involved as well. Although having many of these properties in common with classical solid phase extraction (SPE) the MSPD process is distinctly different, possessing elution and retention properties that appear to be a mix of partition, adsorption and paired ion/paired component chromatography that is somewhat unique. These properties are effected by the following variables. (i) The solid support and the bound phase utilized. (ii) The nature of the sample matrix. (iii) The ratio of sample to solid support. (iv) The solvent elution sequence performed. (v) The use of matrix modifiers. One may influence the disruption, distribution and subsequent elution profile of an MSPD column by blending the sample in the presence of acids, bases, salts, chelators, preservatives or other modifiers. (vi) The use of various solid support combinations or tandem column configurations. We have observed that for many of the drugs and matrices examined, little or no further clean-up or chemical manipulation of the sample is necessary following elution. However, several classes of compounds co-elute with sample matrix components that interfere with detection or that foul the instrumentation after several injections. In some cases a simple back-extraction or re-solubilization process has eliminated such interferences. For several drugs a more efficient process has been the use of tandem columns. For example, the MSPD isolation of nine pesticides from bovine fat or catfish muscle and the fourteen pesticides for oysters and crawfish hepatopancreas is assisted by including in the bottom of the same column 2 g of Florisil, which has little retention for such compounds but readily removes lipids and other materials that adversely effect subsequent GC–electron-capture detection. Similarly, Schenck *et al.* have utilized alumina SPE columns post elution of nicarbazin from chicken liver [9] and muscle [9] and ivermectin from beef liver [3]. We have also observed that the incorporation of up to 1 g of C_{18} in the bottom of an MSPD column prior to addition of the matrix blend can often provide extra fractionation and clean-up of eluates.

These factors come into play whether one is isolating drugs, pollutants or the naturally occurring components of the sample matrix itself. As shown in Table III one is able to account for the entire sample through isolation of eluted components or removal of uneluted components from the column solid support. As with the drugs and pollutants, there is a discernable specificity of elution of sample structural components from the MSPD column. Based on the analyses conducted on the various tissue fractions [25], the elution sequence employed efficiently fractionated triglycerides (hexane fraction) from steroids (DCM fraction), fractionated proteins into several eluates (MeOH > water > ACN > ethyl acetate) and provided a degree of separation of the various carbohydrate and other components of muscle tissue. Connective tissues were neither disrupted by the process nor were they eluted from the MSPD column. Sonication of the

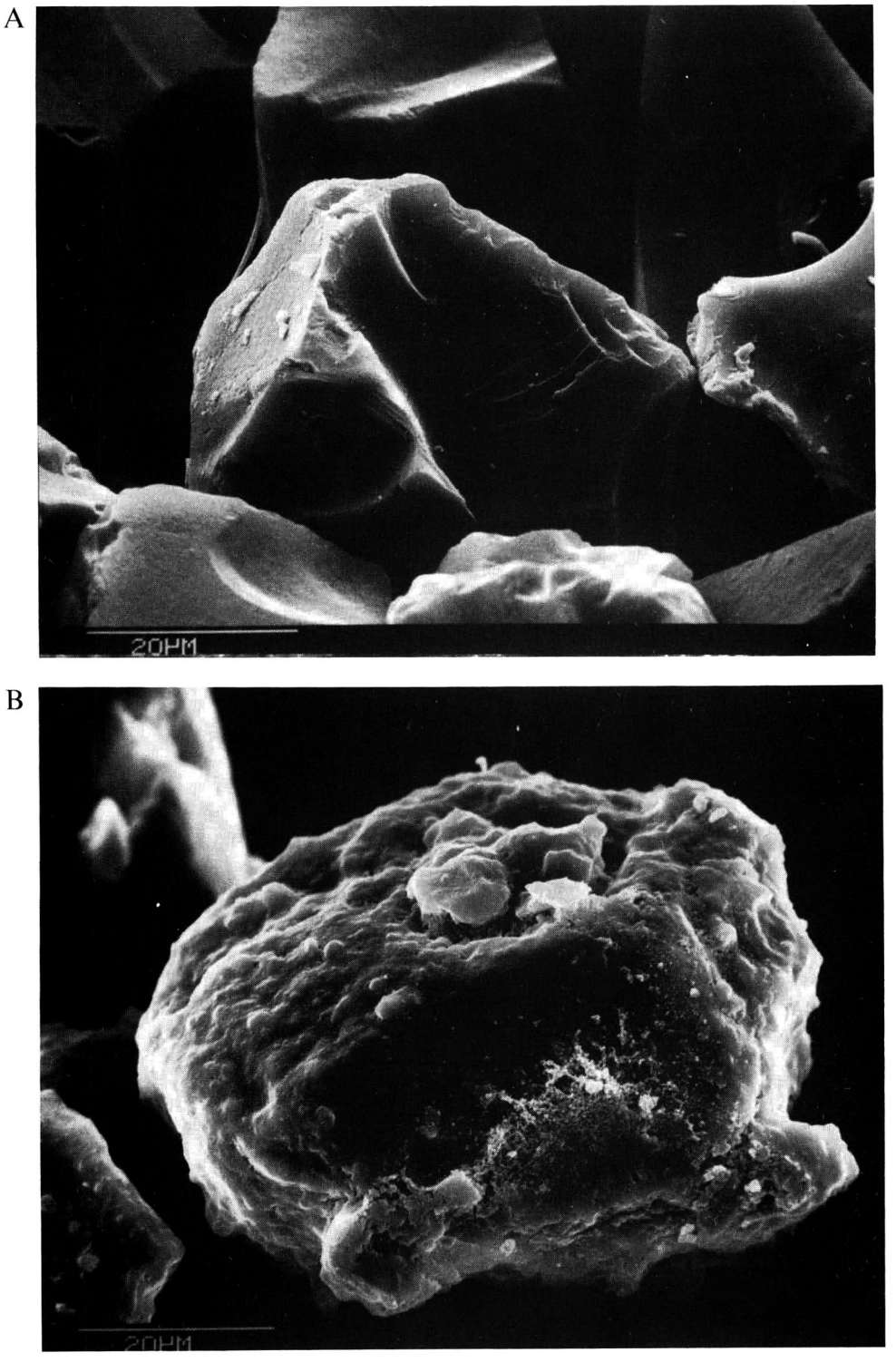
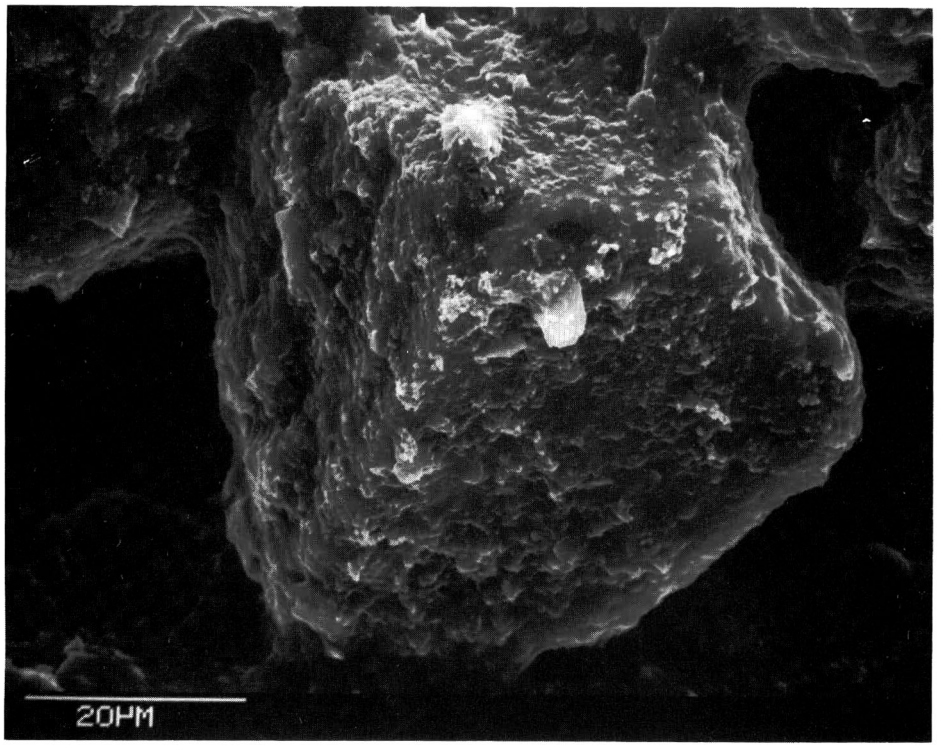


Fig. 1.

C



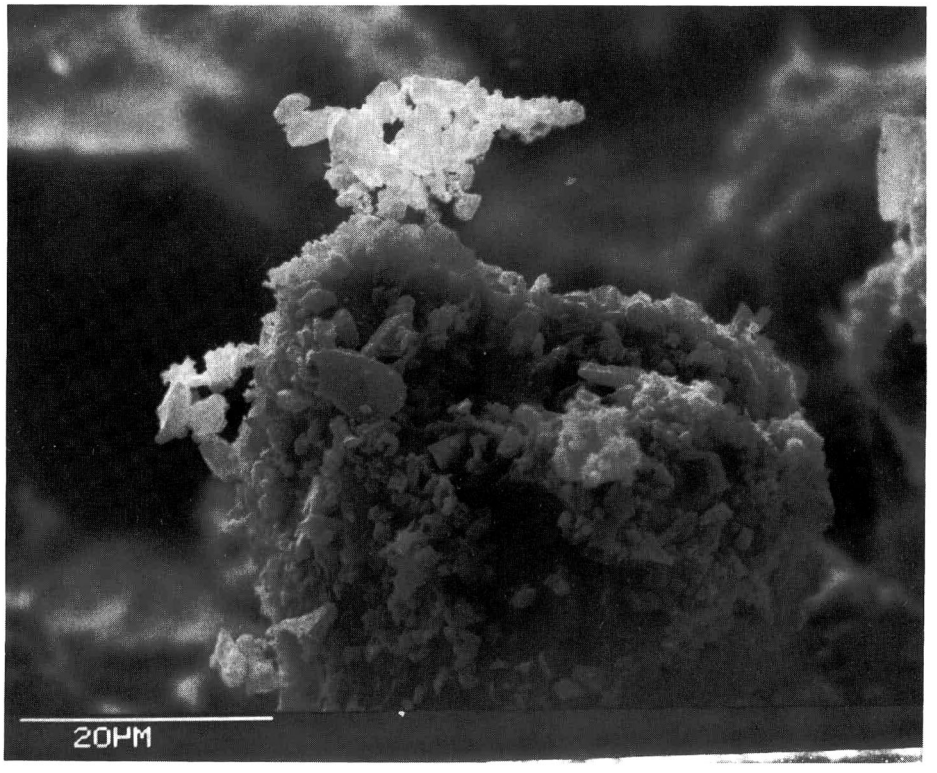


Fig. 1.

(Continued on p. 32)

E

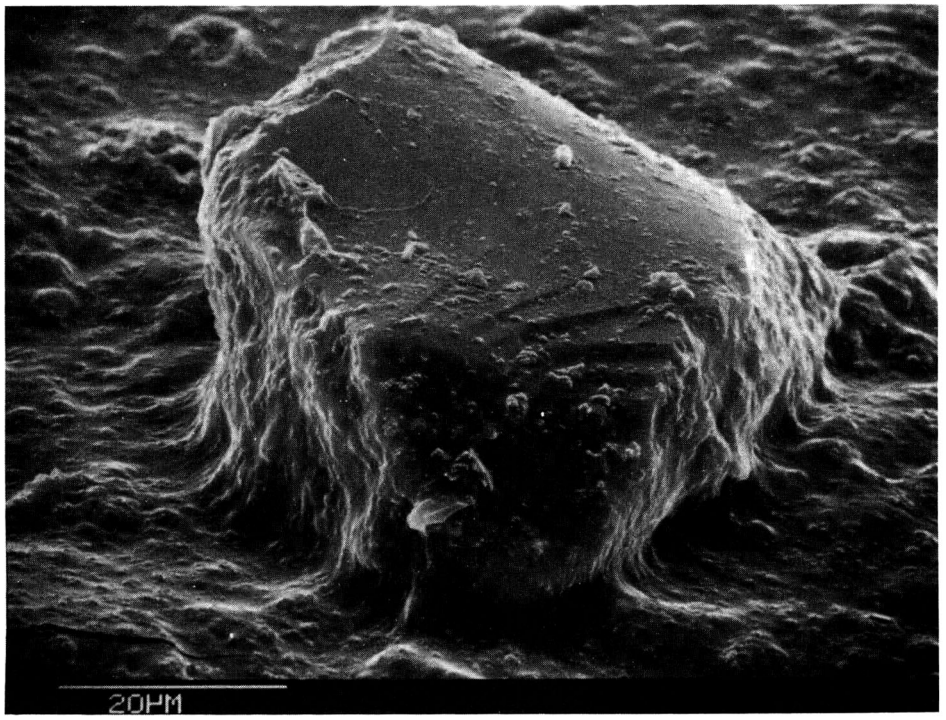


Fig. 1. Scanning electron micrographs of (A) C_{18}-derivatized silica particles used in the studies presented; (B) C_{18}-particles after blending with bovine liver tissue, as described in Experimental; (C) C_{18}-material after blending with pelleted *Mycobacterium paratuberculosis*; (D) underivatized silica particles after blending with *M. paratuberculosis* (Note the degree of silica debris created and the clumping of cells and debris in comparison to B and C); and (E) C_{18}-particle blended with *M. paratuberculosis* showing where two particles had apparently been in contact and broken away. The layer of dispersed material is evident.

column packing allowed isolation of these compounds as an insoluble agglomerate on the surface of the saline sonicate supernatant. Some proteins and materials extractable with a saturated solution of EDTA remained on the column as well but were isolatable by these procedures. The solvent sequence employed no doubt contributed to denaturation of some of the proteins and of larger nucleoside (DNA, RNA) components. Although not examined in the case of muscle tissues we have observed for bacteria that DNA, RNA and other higher-molecular-mass components can be eluted from the less polar components of an MSPD column by beginning the elution sequence with aqueous buffers and reducing the solvent strength as one progresses, *i.e.*, reversing the order of solvent strength elutions performed here. However, the degree of fractionation specificity is greatly diminished [27]. This difficulty may be resolved by including a more polar polymer phase solid support in the blend.

Thus, in the MSPD process for the isolation of drugs or pollutants, one is simultaneously performing a sample matrix fractionation and distinct matrix components will be found to be associated with different drug or pollutant classes. Indeed, the unique elution characteristics of MSPD colums may be directly related to the association of target molecules with the co-eluting tissue components that distribute in the eluting solvents and their interaction with the solid support and the remaining tissue components.

As seen in Table III the application of MSPD to bacteria also afforded a high degree of sample recovery and specificity of elution (Table IV). It should be noted that mycobacteria, such as *M. paratuberculosis*, often require rather severe procedures for complete cellular disruption, such as treatment with antibiotics and high concentrations of detergents or extended sonication at maximum intensity [27]. Indeed, mycobacteria are among the most difficult bacteria to disrupt due to the thick-

ness of their cell walls. *E. coli*, on the other hand, can be readily disrupted by treatment with lysozyme and low concentrations of detergents or short bursts of sonication. However, the results seem to indicate that the MSPD process can perform facile lysis and fractionation of both of these bacterial classes in a chemically and physically mild manner while providing the ability to perform sample fractionation in a single step.

The protein elutions/distributions for *E. coli* (water ≫ MeOH > ACN) and *M. paratuberculosis* (water = DCM > MeOH > ACN) were somewhat different. This may be due to differences in hydrophobicity of certain mycobacterial *versus E. coli* proteins and differences in the interactions between the individual proteins and the remaining constituents on the column. The distribution of proteins suggests that those eluting in the more non-polar solvents (DCM, ACN) may be structurally more lipophilic and possibly associated with cell membranes in their native environment. Further research is being conducted to answer this possibility.

The data also indicate that the MSPD process separated the neutral lipids (hexane fraction) from the phospholipids (DCM and MeOH fractions) in the bacteria. Likewise, fractionation of sterols, indoles, amino acids, purines, pyrimidines, inositols and other mono- and disaccharides was observed for each of the bacteria. However, their individual solvent distributions varied between the two types of bacteria. Since *E. coli* also has a high lipopolysaccharide content each fraction was examined for this compound and was found to reside only in the water fraction. Similarly, nucleic acids and nucleotides were found only in the water fraction but did not account for the total nucleotide content for the mass of cells extracted. We have observed that by beginning the elution sequence with water that a much higher recovery of genomic and plasmid DNA can be obtained from these bacteria and that digestion of the DNA with various restriction endonucleases can be accomplished [27,35].

Taken together these data lend strong support to the proposal that the MSPD process provides a new and generic technique for the homogenization, lysis and/or fractionation of a variety of biological matrices. This conclusion is further borne out by the data obtained from SEM analysis of the various materials. Fig. 1A shows the nature of the solid support/C_{18} particles themselves. The material used is irregular in shape and contains serrated and sharp edges. These characteristics, as in the use of sand, alumina or other abrasives, assist in the initial disruption of the matrix achitecture. Fig. 1B shows the same material after blending with bovine liver. Examination of these and other tissues gave no indication for the existence of intact subcellular structures or individual cells, supporting the idea that complete cellular lysis and dispersion is occurring. Similarly, Fig. 1C shows the result of blending the C_{18} with mycobacteria. Again, no intact cells were noted. This should be contrasted to the results seen with mycobacteria after grinding with identical silica particles that differed only in the fact that they were not derivatized with C_{18} (Fig. 1D). In this case fractured silica particles and clusters of intact mycobacteria were observed. Thus, the use of silica particles alone and the application of shearing forces by grinding were inadequate to obtain lysis of the mycobacteria.

That the cellular components are dispersed is somewhat evident from the various figures wherein C_{18} derivatized particles were used. For example, as shown in Fig. 1E, we often observed particles that had apparently been in contact with others and had broken away. These micrographs show what appears to be a thin layer or film of dispersed material over the surface of the particles. Measurement of the thickness of this layer gave a value of approximately 100 Å.

This approach has several possible advantages over classical methods for the disruption, lysis and fractionation of biological matrices. The process of blending is easy to perform and does not require expensive equipment or special devices. For the types of samples examined the process provides a homogeneous blend of the biological and incurred components dispersed over a large surface area in a thin film. It is applicable to easy or difficult to disrupt tissues as well as bacteria without modification of the process or extra exertion. It is chemically and physically mild, exposing the sample to no excessive heating, mechanical forces or strong chemical or detergent-based reagent. The material obtained can be packed into a column from which compounds may be isolated by various elution schemes. There is sufficient flexibility in the method to allow use of the

listed variables to make the approach applicable and efficient for a variety of needs. It uses, relatively speaking, very small volumes of extracting solvents, greatly reducing solvent need and disposal costs. The method, as applied here, is capable of providing a rapid isolation of the target molecule(s) with a high degree of specificity. The inclusion of co-columns and the use of other column technologies (SPE, immunoaffinity, etc.) could further enhance this specificity while retaining its overall efficiency. The process is also amenable to automation, using an automated mortar and pestle to prepare the samples and eluting the columns using robotics or batch processing instrument configurations. The overall process is also quite rapid and can greatly reduce the time required to isolate and process samples for various needs.

Given these possibilities there remains a wide range of analytical problems to which MSPD may be applicable. However, it is recognized that it will not be applicable in all cases. Nevertheless, if it is truly generic, it will provide an additional method to the analyst for assisting in resolving difficult analytical problems when dealing with biological matrices.

ACKNOWLEDGEMENTS

This work was supported by Cooperative Agreements 5U01-FD-01319, FD-U-000235 and FD-U-000581 with the Food and Drug Administration, United States of America.

REFERENCES

1 S. A. Barker, A. R. Long and C. R. Short, *J. Chromatogr.*, 475 (1989) 353.
2 F. J. Schenck, *Lab. Inf. Bull.*, 7 (1991) 3559.
3 F. J. Schenck, S. A. Barker and A. R. Long, *Lab. Inf. Bull.*, 5 (1989) 3381.
4 A. R. Long, L. C. Hsieh, A. C. Bello, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Agric. Food Chem.*, 38 (1990) 427.
5 F. J. Schenck, S. A. Barker, A. R. Long, *Lab. Inf. Bull.*, 6 (1990) 3426.
6 A. R. Long, L. C. Hsieh, M.S. Malbrough, C. R. Short and S. A. Barker, *J. Agric. Food Chem.*, 38 (1990) 430.
7 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 93.
8 M. M. Soliman, A. R. Long and S. A. Barker, *J. Liq. Chromatogr.*, 13 (1990) 3327.
9 F. J. Schenck, S. A. Barker, A. R. Long and J. Matusik, *Lab. Inf. Bull.*, 5 (1989) 3354.
10 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 868.
11 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 864.
12 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Food Comp. Anal.*, 3 (1990) 20.
13 A. R. Long, M. S. Malbrough, L. C. Hsieh, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 860.
14 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 739.
15 A. R. Long, C. R. Short and S. A. Barker, *J. Chromatogr.*, 502 (1990) 87.
16 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Liq. Chromatogr.*, 12 (1989) 1601.
17 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Agric. Food Chem.*, 38 (1990) 423.
18 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 379.
19 M. D. Crouch and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, (1992) submitted for publication.
20 A. R. Long, M. M. Soliman and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 493.
21 A. R. Long, M. D. Crouch and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 667.
22 H. M. Lott and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, (1992) in press.
23 H. M. Lott and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, (1992) in press.
24 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 813.
25 S. A. Barker and A. R. Long, *Proceedings of the Pittsburgh Conference, New York, 1990*, Abstract No. 859.
26 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
27 M. E. Hines II, A. R. Long, T. G. Snider III and S. A. Barker, *Anal. Biochem.*, 195 (1991) 197.
28 M. A. Markwell, S. M. Haas, L. L. Bieber and N. E. Tolberg, *Anal. Biochem.*, 87 (1978) 206.
29 U. K. Laemmli, *Nature*, 227 (1970) 680.
30 W. Wray, T. Boulikas, V. P. Wray and R. Hancock, *Anal. Biochem.*, 118 (1981) 197.
31 J. R. Dillon, A. Nasim and E. R. Nestermann, *Recombinant DNA Methodology*, Wiley, New York, 1985, p. 13.
32 J. R. Dillon, A. Nasim and E. R. Nestermann, *Recombinant DNA Methodology*, Wiley, New York, 1985, p. 102.
33 Y. Yoshida, E. Furuya and K. Tagawa, *J. Biochem.*, 88 (1980) 463.
34 Y. D. Karkhanis, J. Y. Zeltner, J. J. Jackson and D. J. Carlo, *Anal. Biochem.*, 85 (1978) 595.
35 M. E. Hines II, J. M. Jaynes, S. A. Barker, J. C. Newton and T. G. Snider III, *Infect. Immun.*, (1993) in press.

Journal of Chromatography, 629 (1993) 35–40
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 415

High-performance liquid chromatography *versus* solid-phase extraction for post-derivatization cleanup prior to gas chromatography–electron-capture negative-ion mass spectrometry of N1,N3-bis-(pentafluorobenzyl)-N7-(2-[pentafluorobenzyloxy]ethyl)xanthine, a product derived from an ethylene oxide DNA adduct

Manasi Saha and Roger W. Giese

Department of Pharmaceutical Sciences in the College of Pharmacy and Allied Health Professions, and Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, Boston, MA 02115 (USA)

ABSTRACT

N7-(2-Hydroxyethyl)xanthine (N7-HEX), as a standard, has been measured at the low picomole level by the following sequence of steps: (1) derivatization with pentafluorobenzyl bromide; (2) post-derivatization sample cleanup by reversed-phase high-performance liquid chromatography (HPLC) or silica solid-phase extraction; and (3) separation/detection by gas chromatography electron-capture negative-ion mass spectrometry (GC–ECNI-MS). The average yield of product from the two sample cleanup procedures applied to 95 pg (0.48 pmol) of N7-HEX was comparable: 60% for HPLC; 56% for solid-phase extraction. The reaction blanks (0 pg N7-HEX) showed an interfering GC–ECNI-MS peak after HPLC cleanup. This problem was not encountered with solid-phase extraction, which, along with its greater convenience, made it the preferred technique for post-derivatization sample cleanup.

INTRODUCTION

We are pursuing the detection of DNA adducts in physiological samples as biomarkers for human exposure to toxic chemicals. A DNA adduct is a site on the DNA where a toxic chemical has caused covalent damage. Many carcinogens and mutagens appear to act by producing DNA adducts [1]. High sensitivity is required to measure DNA adducts in physiological samples since the samples not only contain little DNA, but small numbers of such adducts may be toxic. The techniques which are being employed or developed for this purpose have been reviewed [2].

Previously we reported our initial progress on the detection of N7-(2-hydroxyethyl)guanine, an ethylene oxide DNA adduct, by gas chromatography–electron-capture negative-ion mass spectrometry (GC–ECNI-MS) [3]. In order to make this adduct sensitive for detection by GC–ECNI-MS, we converted it, as a standard at the milligram level, to N7-(2-hydroxyethyl)xanthine (N7-HEX) with nitrous acid, followed by derivatization with pentafluorobenzyl bromide to afford N1, N3-bis-(pentafluorobenzyl)-N7-(2-[pentafluorobenzyloxy]ethyl)xanthine, $(PFBz)_3$-N7-HEX. As little as 1.3 attomoles [signal-to-noise ratio (S/N) = 10] of the latter, as a diluted standard, can be detected by GC–ECNI-MS [4].

Correspondence to: Professor R. W. Giese, 122 Mugar Building, Northeastern University, Boston, MA 02115, USA.

0021-9673/93/$06.00

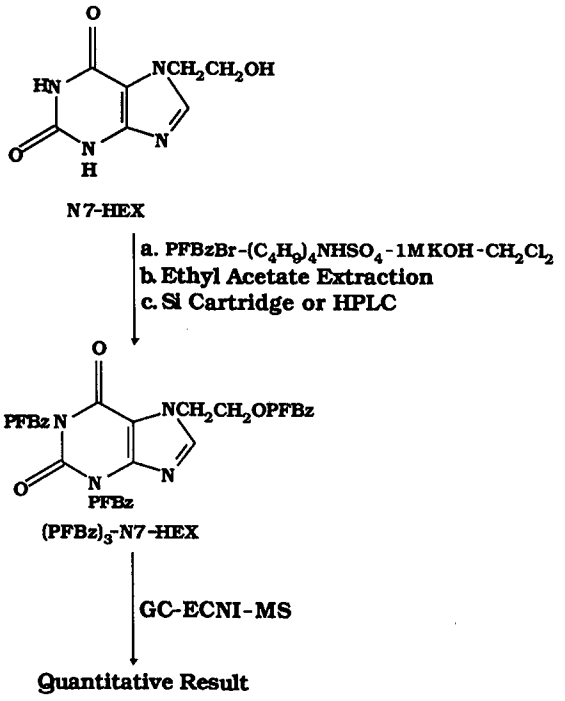


Fig. 1. Scheme for the detection of N7-HEX by GC–ECNI-MS.

Here we report additional progress towards the goal of detecting a trace amount of N7-(2-hydroxyethyl)guanine in a physiological sample. We have been working on the pentafluorobenzylation reaction (Fig. 1), and now are able to conveniently detect as little as 95 picograms (0.48 picomole) of standard N7-HEX by electrophore derivatization–post-derivatization sample cleanup–GC–ECNI-MS, as reported here. This progress has been made largely by focussing on the post-derivatization sample cleanup step. The derivatization reaction, even when performed in the absence of analyte, yields many interfering products that need to be removed prior to injection of the sample into the GC–ECNI-MS system.

EXPERIMENTAL

Reagents

N7-(2-Hydroxyethyl)xanthine was prepared as described [3]. Pentafluorobenzyl bromide, potassium hydroxide and tetrabutyl ammonium hydrogensulphate were purchased from Aldrich (Milwaukee, WI, USA). HPLC grade organic solvents were purchased from Doe and Ingalls (Medford, MA, USA).

High-performance liquid chromatography (HPLC)

A Microsorb silica reversed-phase column, 150 × 4.6 mm I.D., 10 μm (Rainin, Woburn, MA, USA) was used. The mobile phase was acetonitrile (ACN)–water (65:35, v/v) at 1 ml/min. Detection at 270 nm was done with a Spectra Monitor 3000 (LDC-Milton Roy, Riviera Beach, FL, USA) and an SP4270 integrator (Spectra-Physics, San Jose, CA, USA).

GC–ECNI-MS

The GC (5890 Series II), MS (5900A) and data system (59970 MS Chemstation) were from Hewlett-Packard (Palo Alto, CA, USA). Methane (2 Torr) and helium (20 p.s.i.) were used as reagent and carrier gases, respectively. Other conditions were as described before [4]. Specially cleaned and silanized glassware, pipettes and syringes were used to minimize adsorption losses and cross contamination [5].

Solid-phase extraction

The solid-phase extraction columns were prepared by dry-packing 200 mg of silica gel (60-Å pore, 40-μm irregular particles, J. T. Baker, Phillipsburg, NJ, USA) into a 5.25-in. borosilicate Pasteur pipette containing silanized glass wool (J. T. Baker).

Derivatization of N7-HEX

N7-HEX (95 pg, 0.48 pmol) in 10 μl of 1 *M* HCl was evaporated in a vial under nitrogen. From a stock solution of tetrabutyl ammonium hydrogensulphate (5 mg in 5 ml of 1 *M* KOH) was added 50 μl (0.15 μmol) to each vial with subsequent addition of 150 μl of CH_2Cl_2 and 10 μl (0.065 μmol) of pentafluorobenzyl bromide. The reaction mixture was stirred at room temperature for 20 h and the residual CH_2Cl_2 was slowly evaporated under nitrogen. Water (50 μl) and ethyl acetate (150 μl) were added, and the organic layer after vortexing and centrifugation was collected. Three more 150-μl ethyl acetate extractions were done, and the combined organic layer was evaporated under nitrogen, redissolved in 50 μl of acetonitrile prior to HPLC,

or in 50 μl of hexane–ethyl acetate (1:1, v/v) prior to solid-phase extraction, for post-derivatization sample cleanup as presented in Table I, followed by GC–ECNI-MS as also described in this table.

RESULTS AND DISCUSSION

For post-derivatization sample cleanup of $(PFBz)_3$-N7-HEX prior to detection by GC–ECNI-MS, we decided to explore both HPLC and solid-phase extraction. HPLC is attractive for this purpose because of its speed, high resolution, availability of automated equipment, the high quality of modern HPLC packings, and the use of stainless-steel hardware. These features collectively could be important in overcoming the classic, two general problems in trace organic analysis: losses and interferences. However, since all samples traverse the same HPLC system, it can be necessary to thoroughly clean the system between injections to minimize sample-to-sample effects such as carryover (ghosting) of the analyte. Below a certain level of analyte, on-line UV detection is no longer possible, so any shifts in retention of the trace analyte might be a second difficulty.

These two concerns about sample cleanup by HPLC prior to detection by GC–ECNI-MS should be easily avoided by solid-phase extraction. A new cartridge for each sample eliminates the difficulty of sample ghosting, and the use of optimized washing and elution conditions can assure the location of the analyte for a given batch of packing as long as the sample matrix is relatively constant. On the other hand, it is not clear that packings for solid-phase extraction are manufactured with as much attention to quality and reproducibility as those for HPLC. Also, solid-phase extraction is a lower-resolution technique. Thus we wanted to explore both HPLC and solid-phase extraction.

For HPLC, we selected a C_{18}-silica packing because of the well-deserved popularity of this type of column, particularly its resistance to contamination by conventional, small-molecule samples, and the speed and ease of achieving re-equilibration of the column after solvent changes. The latter property is important for the thorough washing of the HPLC system that we anticipated to be important for our trace samples.

The HPLC procedure that we developed for sample cleanup of $(PFBz)_3$-N7-HEX derived from subjecting 95 pg (0.48 pmol) of N7-HEX to our derivatization reaction is summarized in Table I. This procedure was the outcome of preliminary experiments (data not shown) that led to the conditions selected. Obviously the procedure is very tedious, particularly the extensive washing of the column

TABLE I

TWO PROCEDURES USED FOR POST-DERIVATIZATION SAMPLE CLEANUP

Double HPLC (C_{18}-silica)

(1) Inject 29 ng of standard $(PFBz)_3$-N7-HEX into the HPLC system to define the retention time *e.g.* 9.0 min for this compound (UV detection).
(2) Clean the HPLC system.[a]
(3) Inject 50 μl of acetonitrile (ACN blank A), collect 2 ml, and hold for steps 11, 12. Repeat HPLC cleaning and ACN injection twice (ACN blanks B, C).
(4) Inject reaction blank A, collect 2 ml, and hold for steps 11 and 12.
(5) Clean the HPLC system.[a]
(6) Repeat (steps 4 and 5) for reaction blank B, and for reaction blank C.
(7) Inject reaction sample A, collect 2 ml and hold for steps 11 and 12.
(8) Clean the HPLC system.[a]
(9) Repeat for reaction sample B, and for reaction sample C.
(10) Clean the HPLC system.[a]
(11) Evaporate ACN blank A, redissolve in 50 μl ACN, inject into the HPLC system, collect 2.0 ml, evaporate, redissolve in 50 μl of toluene, and inject 1 μl into the GC–ECNI-MS system.
(12) Wash the HPLC injector with 3 ml of hot ACN (as in step 2).
(13) Repeat steps 11 and 12 for each of the remaining blanks and samples.

Solid-phase extraction

(1) Wash the silica cartridge with 1 ml of ethyl acetate and 1 ml of hexane.
(2) Load the reaction blank (A, B) or reaction sample (A, B, C, D) in 50 μl of hexane–ethyl acetate (50:50, v/v).
(3) Wash with 4 ml of hexane and 8 ml hexane–ethyl acetate (90:10, v/v).
(4) Elute with 2 ml of ethyl acetate.
(5) Evaporate and continue as in above step 10.

[a] The HPLC column was cleaned by twice conducting a gradient from ACN–water (65:35, v/v) to 100% ACN over a 10-min period, holding for 4 min, and returning over a 10-min period to the initial condition. Before each gradient wash, the injector was washed with 0.5 ml of warm ACN in the inject position and the same in the load position.

and injector between injections, and the separation of each sample twice by HPLC. But our earlier experiments gave irreproducible results by GC–ECNI-MS in terms of both losses and interferences when less rigorous inter-sample washing of the HPLC system was done, or when the samples were subjected to a single HPLC separation.

The results of this procedure (Table I) are shown in Table II. Remarkably, once 29 ng of $(PFBz)_3$-N7-HEX are injected into the HPLC system in order to establish the retention time (with UV detection), extensive washing of the HPLC system is necessary before the compound is completely removed from this system. As seen, the first two ACN blanks (A and B; pure acetonitrile is injected) become contaminated [2.9 and 3.9 pg of apparent $(PFBz)_3$-N7-HEX, respectively, at the end of the overall procedure]. Finally the third ACN blank (C), collected after the column and injector have been subjected to a 3-h cleaning procedure (defined in Table I), shows no analyte, as seen in Table II.

For the reaction blanks A–C, it is not clear whether the observed, interfering peak by GC–ECNI-MS, as cited in Table II, is contaminating analyte or some other interfering compound derived from the derivatization reaction. Conceivably the HPLC system is still contaminated by analyte from the earlier, 29 ng injection, and the injection of a reaction matrix as opposed to injection of pure-solvent (the ACN blanks) displaces additional analyte from the system. At least the reaction itself is working at this level: the reaction sample A–C show an amount of product which is significantly higher than that present in the reaction blanks. If we subtract the average value for the reaction blanks from the average value for the reaction samples, we obtain a yield for the reaction of 60%. A representative GC–ECNI-MS chromatogram for a reaction blank is shown in Fig. 2a, and for a reaction sample in Fig. 2b. Note that the abundance scales are different, so the amount of the interfering peak (Fig. 2a) relative to the product peak (Fig. 2b) is different than the visual appearance. While we could have explored the origin of the contamination in more detail, we were disuaded from this by the much better performance provided by solid-phase extraction for sample cleanup, that we investigated in parallel, as presented below.

TABLE II

DETERMINATION OF N7-HEX BY PENTAFLUOROBENZYLATION–GC–ECNI-MS USING THE TWO POST-DERIVATIZATION SAMPLE CLEANUP PROCEDURES PRESENTED IN TABLE I

Sample cleanup procedure	Amount of $(PFBz)_3$-N7-HEX or interference[a] (pg) starting from 0 pg (ACN blanks and reaction blanks) or 95 pg (reaction samples) of N7-HEX. A 100% yield of product would be 356 pg.
Double HPLC (C_{18}-silica)	
ACN blanks (A,B,C)	2.9, 3.9, 0.0
Reaction blanks (A,B,C)	4.1, 10.5, 29.0
Reaction samples (A,B,C)	205, 305, 129
Solid-phase extraction (silica)	
Reaction blanks	0,0
Reaction samples (A,B,C,D)	260, 180, 124, 234

[a] The interference is quantified, although it is an unknown, by assuming that it is $(PFBz)_3$-N7-HEX.

For post-derivatization sample cleanup by solid-phase extraction, we selected a silica packing. While C_{18}-silica packings are also popular for solid-phase extraction, we wanted to avoid the potential for variability in the C_{18} bonding to silica by manufacturers. Also, we had previously found a silica cartridge to be useful for post-derivatization sample cleanup of an electrophoric derivative of 5-methylcytosine prior to detection by GC with electron-capture detection [6].

The convenient procedure that we developed for post-derivatization sample cleanup of $(PFBz)_3$-N7-HEX by solid-phase extraction on a silica cartridge is summarized in Table I. After the samples are loaded onto the cartridge in hexane–ethyl acetate (50:50) washing is done with hexane–ethyl acetate (90:10), and then product is eluted with ethyl acetate. The results from subsequent measurement of the reaction blanks and reaction samples by GC–ECNI-MS are shown in Table II. As seen, no interfering peaks are encountered in the reaction blanks, unlike what was observed after sample cleanup by HPLC. The average yield of product from the four reaction samples tested (A–E) is 56%. [In prior development work, spiking 57 pg in duplicate of $(PFBz)_3$-N7-HEX into a reaction blank followed by solid-phase extraction–GC–ECNI-MS gave a recovery of 84 and 85%.] Obviously this technique

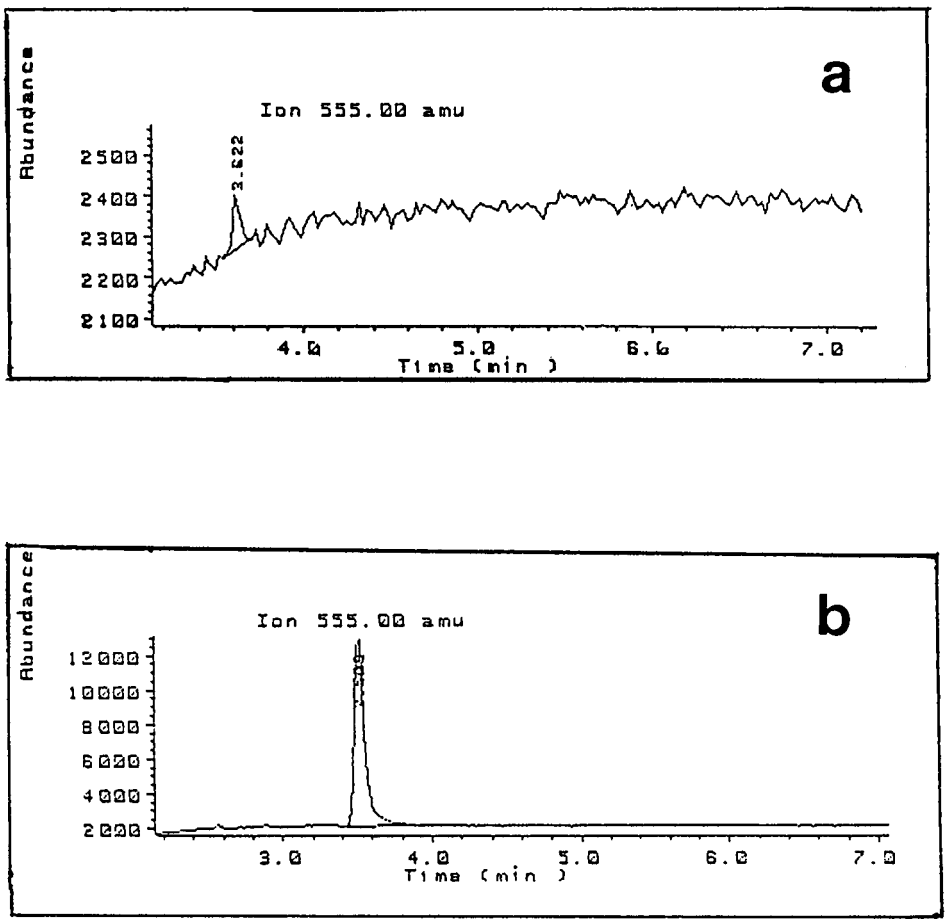


Fig. 2. Detection of $(PFBz)_3$-N7-HEX by GC–ECNI-MS obtained via the scheme shown in Fig. 1 and using double HPLC for post-derivatization sample cleanup as detailed in Table I. (a) Reaction blank A (see Table II); 0 pg of N7-HEX was derivatized. (b) Reaction sample B (see Table II); 95 pg of N7-HEX was derivatized. For both chromatograms, 1/50 of the final sample in 50 μl of toluene was injected. Each peak shown co-elutes with authentic $(PFBz)_3$-N7-HEX. GC column length: 12 m.

wins over the tedious, contaminant-prone double HPLC technique. A representative GC–ECNI-MS chromatogram for a reaction blank is shown in Fig. 3a, and for a reaction sample in Fig. 3b, after silica solid-phase extraction.

CONCLUSION AND FUTURE WORK

A convenient procedure utilizing silica solid-phase extraction for post-derivatization sample cleanup has been developed for the detection of standard N7-(2-hydroxyethyl)xanthine at the low picomole level by GC–ECNI-MS. Since the yield and signal-to-noise ratio are high, there is no interfering peak in the reaction blank, and only 1/50 of the final sample is injected into the GC–ECNI-MS, potentially the technique can be taken to a lower analyte level. We will be pursuing this goal in our future work, and also attempting to extend the method to the detection of a trace amount of the parent adduct, N7-(2-hydroxyethyl)guanine, in biological samples. It is desirable to reach at least the femtomole level in order to minimize the amount of such samples required.

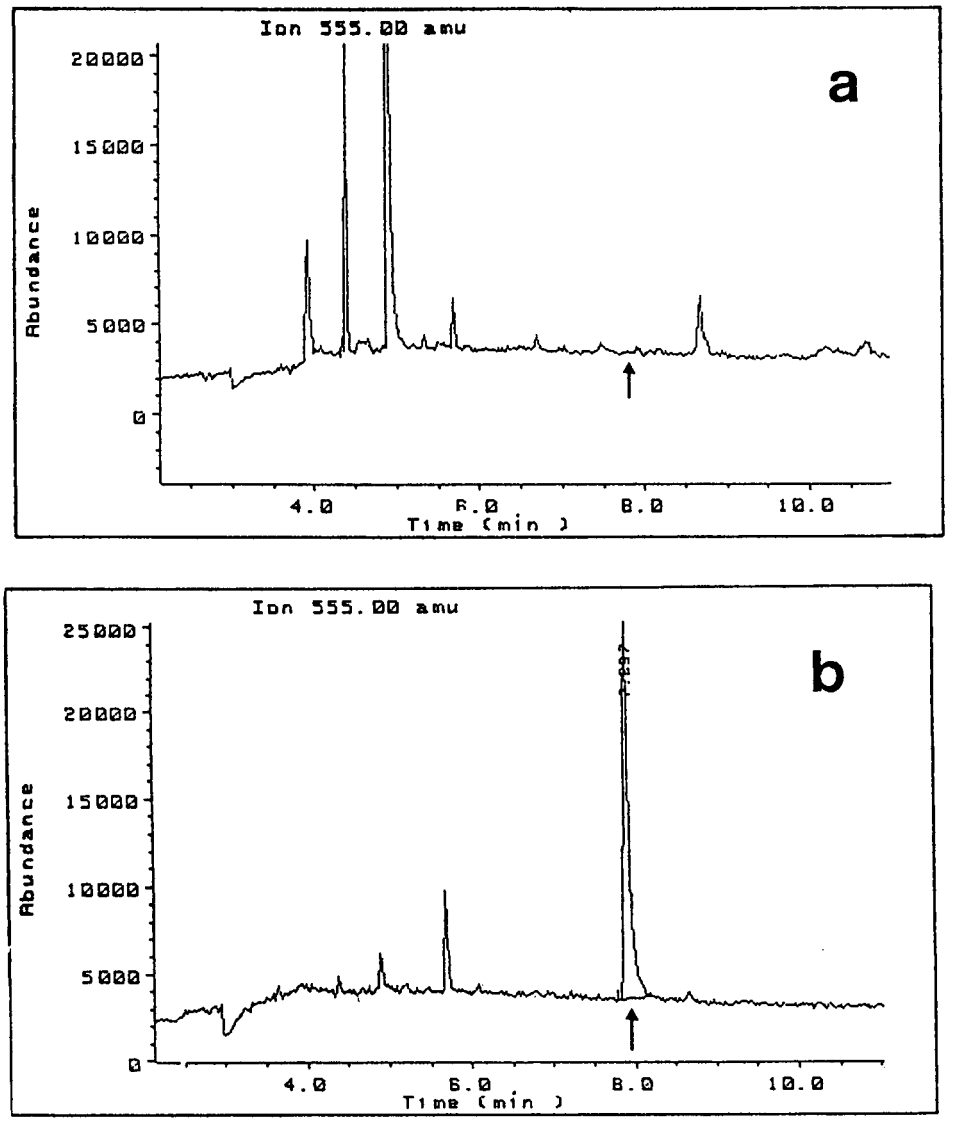


Fig. 3. Same as Fig. 2 except silica solid-phase extraction was used for post-derivatization sample cleanup (see Table II). (a) Reaction blank. (b) Reaction sample A (see Table II). The peak or retention position (based on injecting authentic product) for $(PFBz)_3$-N7-HEX is shown with an arrow. GC column length: 20 m.

ACKNOWLEDGEMENT

This work was supported by Grant OH02792 from the National Institute for Occupational Safety and Health, Centers for Disease Control. Contribution No. 537 from the Barnett Institute of Chemical Analysis.

REFERENCES

1 B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, 1983.
2 R. C. Garner, P. B. Farmer, G. T. Steel and A. S. Wright (Editors), *Human Carcinogen Exposure*, IRL Press at Oxford University, Press, Oxford, 1991.
3 K. Allam, M. Saha and R. W. Giese, *J. Chromatogr.*, 499 (1990) 571.
4 S. Abdel-Baky and R. W. Giese, *Anal. Chem.*, 63 (1991) 2986.
5 J. Adams, M. David and R. W. Giese, *Anal. Chem.*, 58 (1986) 345.
6 D. H. Fisher and R. W. Giese, *J. Chromatogr.*, 452 (1988) 51.

Journal of Chromatography, 629 (1993) 41–53
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 673

Solid-phase sample preparation of natural waters with reversed-phase disks

Theresa McDonnell and Jack Rosenfeld

Department of Pathology, Faculty of Health Sciences, McMaster University, Hamilton, Ontario, L8N 3Z5 (Canada)

Arman Rais-Firouz

National Laboratory for Environmental Testing, Canada Centre for Inland Waters, Burlington, Ontario L7R 4A6 (Canada)

ABSTRACT

An investigation of sample preparation for natural waters using the Empore disk was conducted. The Empore disk is a new solid-phase sample preparation technology which was developed for rapid isolation of organic contaminants from aqueous matrix. In order to increase the volume of water that could be prepared, it was found that in-line or off-line filtration prior to the extraction step was required. The appropriate filters were identified. When more than 10 liters of natural water were analyzed a non-specific interference to capillary gas chromatography–electron-capture detection determination of analytes was present. The use of the Empore disks offered some advantages in ease and specificity of elution that was not available with solvent extraction and other solid-phase sample preparation technologies.

INTRODUCTION

Assessment of risk from organic chemicals in natural waters requires determination of numerous analytes from diverse and complex matrices. Such matrices vary with geography and within a geographical location the matrix can vary with depth of the water column. In a given location the matrix can also vary with time as a result of local and/or upstream weather and seasonal change. Finally the matrix can vary as a result of human activities. Moreover, at this point in time it is still unclear which compounds constitute any given ecological problem. There is, however, sufficient evidence that organic chemicals in water do pose a real ecological hazard. Methods are, therefore, required to permit a thorough investigation of the nature of compounds that put the environment at risk.

Analytical methods that are to be applied to resolution of these problems should have a number of characteristics. Simplicity and rapidity are obligatory as these permit analysis of the large number of samples necessary for appropriate risk analysis. Some applications require independent determination of organics present in the water and those associated with the particulates, whereas for other applications determination of total organics in the sample is sufficient.

The sample preparation step of analytical methods is the most time consuming and most difficult to automate. One approach to simplification, reduction of costs and automation is solid-phase sample preparation (SPSP). The standard configuration for an SPSP apparatus is that of a semi-preparative column or cartridge packed with reversed-phase chromatographic support [1–6]. A more recent SPSP material is based on reversed-phase particles en-

Correspondence to: J. Rosenfeld, Department of Pathology, Faculty of Health Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada.

0021-9673/93/$06.00

meshed in a PTFE network that is configured as a disk [7,8]. The disk permits a faster flow-rate by increasing the cross-sectional diameter of the stream. Efficient adsorption from the fast flowing water is a result of: (i) reduction in linear velocity of the water flow; (ii) reduction of the particle size to 10 μm; (iii) packing these particles densely so that the mean free path of dissolved analyte to an adsorptive surface is small.

While the Empore disk has been used successfully in a number of applications, to date, there has been no extensive study regarding the feasibility of using these disks for the preparation of larger volumes of samples from natural waters. As part of our programme in monitoring contamination of the Great Lakes of North America we investigated determination of analytes concentrated from 10 or more litres of water. Isolation and analysis of organics in more than 10 000–20 000-fold concentration from water is a requirement for this monitoring programme.

Monitoring of natural waters requires that methods of sampling be applicable to samples of differing complexity with some samples being free of particulates and others having a very heavy particle load. A second requirement is that work-up procedures can separate analytes of choice from contaminating materials. We investigated methods for: reducing the deleterious effects of particulates; increasing volume of sample that could be extracted by Empore disks; improving selectivity of sample preparation.

EXPERIMENTAL

Chemicals

Reversed-phase disks with octylsilica (C_8) and octadecylsilica (C_{18}) enmeshed in PTFE were supplied by Dr. C Markell, 3M Corporation (St. Paul MN, USA) or purchased from Varian Associates (Georgetown, Canada). The National Laboratory for Environmental Testing (NLET) at the Canadian Centre for Inland Waters (CCIW) (Burlington, Canada) supplied a solution containing standard organochlorine contaminants in methanol which was used for spiking water. Niagara River water was supplied courtesy of the Water Quality Branch (Ontario Region) and the NLET. Samples from Hamilton Harbour were supplied by the Laboratory of Dr. K.L.E. Kaiser of CCIW and were collected were collected in July 1991 and October 1991. Water was stored at ambient pH and at 4°C; samples collected in July were analyzed within 2 weeks whereas the October samples were stored and analyzed used over a period of 5 months. Extracts from the October samples were stored in dichloromethane at 4°C; this extract was also made available courtesy Dr. K. Kaiser. It was not possible to analyze the CH_2Cl_2 extracts and the Empore disk extracts simultaneously because samples were collected and prepared by liquid–liquid extraction as part of a routine monitoring programme. Timing of collection for this study could not be deferred to compensate for research and development on the use of Empore disks as this would have required collection of samples during the winter during which time the Harbour is frozen.

Diethyl ether, 2,2,4-trimethylpentane (isooctane), hexane and dichloromethane were all analytical grade and purchased from BDH (Toronto, Canada). *n*-Pentane was purchased from Caledon Labs. (Georgetown, Canada). Individual standards were purchased from the National Water Research Institute as solutions of calibrated concentrations in isooctane. Pre-filters used included the following: nylaflo (nylon 6 fibres) 0.2 μm and 0.45 μm (Gelman Sciences, Ann Arbor, MI, USA); multigrade glass microfibre filters (GMF) both 1 μm and 2 μm, 47 mm and 90 mm were from Whatman (Maidstone, UK). The Whatman glass microfibre filter GF/A, 1.6 μm, 90 mm was obtained courtesy of Dr. C. Markell, 3M Corp.

Sampling sites and techniques

The origin of all samples obtained was the western region of Lake Ontario which is the lowest of the Great Lakes.

The Niagara River site. The Niagara River flows from Lake Erie and enters Lake Ontario on the south western shore. Samples from this river were collected at the exit of the river into Lake Ontario at the Niagara-on-the Lake station in July 1991 as part of a monitoring programme of Environment Canada. According to the monitoring protocols established by Environment Canada, samples from the Niagara River were centrifuged by a Westphalia centrifuge prior to liquid–liquid extraction of organics from the water so clarified. This removed particulates greater than 10 μm.

Tap water. Tap water was obtained at the McMaster University Medical Centre which is supplied by the Hamilton municipal water supply. The intake pipes for this water supply is in Lake Ontario is approximately 1 km from the western shore of the lake. Water was collected directly from the tap as needed.

Harbour water. Hamilton Harbour is a partially enclosed water and the westernmost region of Lake Ontario. On the southwest, the harbour is bounded by the city of Hamilton, which is heavily industrialized. A substantial portion of the harbour front is occupied by large steel making installations. On the northwest, the harbour is bounded by a natural swamp fed by waters from a farming area and by runoff from a large open botanical garden. On the east the harbour is bounded by a large sand bar which has a ship channel connecting the harbour to Lake Ontario. Water at this site was collected without any pre-treatment and was sampled at the approximate geographical centre of the harbour.

Instrumentation

Gas chromatographic (GC) analyses were performed on a Hewlett-Packard 5790 gas chromatograph equipped with a capillary column and used under the following conditions: the column was purchased from Supelco (Mississauga, Canada) and was 30 m × 0.32 mm inner diameter, the phase was DB-5 with 0.25 μm film thickness. For the majority of analysis, the initial temperature was 130°C and was raised at 4°C/min to a final temperature of 290°C. The pressure of the hydrogen carrier gas was 9 p.s.i. (1 p.s.i. = 6894.76 Pa) and the linear velocity was 0.62 m/s at 160°C. The flow-rate for argon–methane make-up gas was 48 ml/min. Detection was by electron-capture detection (ECD) with the output recorded on a 3392A Hewlett-Packard integrator at an attenuation of 3 or 4.

Procedure

The SPSP procedure is presented in schematic form in Table I and consisted of two basic steps: filtration/extraction and elution.

Filtration. A standard filtration apparatus was used [7], which was connected to a water aspirator (less than 20 p.s.i.). If the water was to be filtered prior to extraction of the analytes onto an Empore disk, the filter and disk were arranged in one of two configurations. In the on-line configuration, the filter was placed above and in direct contact with the Empore disk, and the water was passed through the combined filters. In the off-line configuration water was filtered through the pre-filter and the filtrate was collected: this filtrate was then passed through the Empore disk.

TABLE I

SIMPLIFIED SOLID-PHASE EXTRACTION METHOD WITH EMPORE MEMBRANES

Prepare membrane by wetting with methanol
Adsorb analytes onto membrane from environmental water
Dry the membrane placing in dessicator for 1 hour
Elute analytes by soaking the membrane in 5 ml of diethyl ether for 10 min and washing with two 2.5-ml aliquots of ether
Concentrate analytes by evaporating ether under a stream of nitrogen to 2 ml. Add 0.5 ml of isooctane containing external standard: evaporate to 0.5 ml of isooctane.
Analyze 1-μl aliquot of extract by GC–ECD
Calculate yields

Isolation of analytes from the extraction disk. The disk was placed in a desiccator containing $CaCO_3$ for 1 h. This eliminated the need to dry the solvents after eluting from the disk and prior to evaporation and concentration. Analytes were eluted from the 47-mm disk by placing the disk in a beaker and leeching the disk with 5 ml of diethyl ether for 20 min. The disks were then washed with two aliquots of 2.5 ml diethyl ether. All eluates were combined. For the 90-mm disk the volumes increased to 20 ml of diethyl ether for the leeching step and to 5 ml for the subsequent washing steps. To the combined ether eluates either 0.5 or 1.0 ml of isooctane was added as a keeper solvent; the isooctane contained 0.1 μg/ml of pentafluorobenzyl nonadecanoate as an unextracted internal standard. The solution was concentrated to 0.5 or 1.0 ml of isooctane under a stream of nitrogen at 30°C, and a 1 μl aliquot was injected directly onto the capillary column.

Spiking of standards into water. Standards were added to a volume of water and stirred for 2 h before extraction. Spiking for determination of recoveries from all matrices were determined at 1 μg/l concentrations.

Calculation of recoveries. For the extracts containing organochlorines, the ratio ($R1$) of the area of each component to the area of a known amount

of pentafluorobenzyl nonadecanoate was determined for each component. A same amount of pentafluorobenzyl decanoate in toluene was added to the spiking solution and the ratio was calculated for each component in the spiking solution ($R2$). To calculate yields, the ratios were compared and reported as a percentage $R1/R2 \times 100$.

Recoveries of 2,4-dichlorophenoxy acetic acid (2,4-D) and 2,4-dichlorophenol were based on the recovery of ^{14}C-labelled analogues. An aliquot of the 30 000 counts radiolabelled compound (equivalent to 0.83 nmol) was added to 1 l tap water, which had been previously adjusted to pH 2.2 with HCl. The spiked water was then filtered through the disk; the disk was not dried, but was extracted immediately with 5 ml of methanol and a 1-ml aliquot was transferred to a counting vial after 10 min. *NOTE: Drying of the disk was not used in this experiment because the analytes are volatile. For this reason, in order to minimize possible losses due to sample preparation, the analytes were eluted from the disk with methanol which could be mixed directly with the scintillation fluid for counting.* A 10-ml volume of scintillation cocktail (Ready Safe, Beckman, Fullerton, CA, USA) was added and radioactivity was determined by scintillation counting.

Fractionation. Isolation of organochlorine compounds from 10–11 l of Hamilton Harbour water required an additional purification step preparatory to determination by capillary GC–ECD. This was due to the presence a non-specific interference (NSI) to such determination that appeared to be related to a yellow coloured material that co-eluted from the adsorption disk with this class of analytes in the ether extract.

Two techniques were studied in order to separate analytes from the coloured material and/or the NSI. In the first, the organic analytes in the diethyl ether eluate from the Empore disk were first transferred to iso-octane during the concentration stage. This isolate was then transferred to a Florosil column consisting of 2 g of Florisil packed into a silanized glass pipette sealed with a silanized glass wool plug. The organics were then eluted with increasing concentrations of diethyl ether in isooctane or *n*-pentane. In the second technique, selective elution from the disk was attempted with the solid phase being leeched with 10, 25 or 50% diethyl ether in *n*-pentane to directly fractionate the yellow material and/or NSI from the organochlorines *in situ*.

RESULTS AND DISCUSSION

Samples and matrices

In order to expand the possible applications of the Empore disks we investigated these disks as an SPSP phase in analysis of large volumes of natural water samples with matrices of varying complexity. The sample with the simplest matrix was obtained at the Niagara Rivers site which had markedly reduced particulate load as a result of centrifugation prior to collection of the water fraction. A somewhat more complex matrix was obtained by using tap water from the Medical Centre of McMaster University; tap water was used without any pretreatment. Samples with higher concentration of particulates were obtained by collecting water from Hamilton Harbour at two different seasons; mid-summer (July) and mid-fall (October). The sample collected in July was turbid indicating an elevated particulate load and the sample collected in October was clear indicating a considerable reduction in the amount of particles present.

The investigations focused on the following questions. What is the magnitude and time course in the reduction in flow-rates through an Empore disk resulting from particulates in the sample? How can this reduction in flow-rates be minimized? What are the recoveries that could be achieved? Do natural components affect the subsequent chromatographic analysis and if adverse effects are found how can these be minimized?

TABLE II

THE EFFECT OF WATER SOURCE ON EXTRACTION TIMES USING A 47-mm EMPORE DISK

ND = Not determined.

Water source	Collection time for 1 l (min)	Collection time for 2 l (min)
Niagara River	12	ND
Tap	20	90
Hamilton Harbour (October 1991)	60	840
Hamilton Harbour (July 1991)	300	ND

Effect of matrix on the adsorption step of SPSP with Empore disks

As expected, water that is relatively free of particulates passed through a 47-mm Empore disk quite rapidly (Table II). Sample preparation time for 1 l of Niagara River Water was only 12 min because particulates had been removed by the Westphalia Centrifuge. Preparation of 1 l of tap water required a somewhat longer time because no laboratory treatment had been used to remove any organic or inorganic particulates remaining from treatment for converting lake water into drinking water [7].

Relative to these particulate free waters, the time for passing 1 l of untreated Hamilton Harbour water through a 47-mm Empore disk increased 3- to 15-fold depending on the particulate load. This volume of water collected in the fall of the year could be passed through a 47-mm Empore disk in 60 min (a reasonable time frame) whereas water collected in the summer required 300 min (Table II). With the sample collected from Hamilton Harbour October 1991 preparation of 2 l required more than 14 h to pass through a 47-mm Empore disk and this is prohibitive. (Due to the high particle load, it was not considerable practical to prepare 2 l of sample collected in July 1991). Increase in preparation time for 2 l rather than 1 l (although not as dramatic) was also observed for tap water. The exponential rate in decrease of flow through an Empore disk has been reported [7] and was reproducible in our hands; the relative standard deviation of time for collection of a given volumes on 6 different Empore disks was less than 14% for times of greater than 6 min and less than 18% for times of less than 4 min (see also Table IV).

Development of methods to increase the volume of sample prepared

The exponential curve in Fig. 1 was attributed to progressive occlusion by particulate matter of the disk with increased volume of water passed through the disk. Such a mechanism was also proposed by Hagen *et al.* [7]. Consequently, in order to increase the amount of sample that can be prepared in reasonable times, we investigated filtration prior to the adsorption step on the disk. This approach, rather than acidification [7], was selected because the intent of these studies was to develop methods for the wide diversity of sample types and problems encountered in environmental monitoring and research. In this case it was important to consider a number of factors.

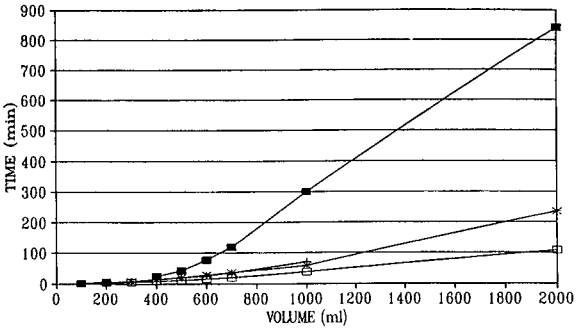


Fig. 1. Hamilton Harbour water collected in October 1991 was extracted under the following conditions: ■ = no filter octylsilica Empore disk alone; + = 0.45 μm filter on-line with octylsilica Empore disk; ✳ = 2.0 μm GMF filter on-line with octylsilica Empore disk; □ = 1.0 μm GMF filter on-line with octylsilica Empore disk.

First, the chemical and physical composition of materials in natural waters that could occlude disk pores is highly variable. These materials can be of inorganic [7], organic or microbiological origin and the use of acidification would only be effective in dissolving the inorganic particulates. Second, although acidification is used as a preservation technique and is indeed obligatory for some analyses (*e.g.* isolation of acid herbicides), lowering of the pH may not be applicable to the wider range of problems. It should be recognized that under acid conditions the inorganic matrix and acid labile organic compounds are destroyed. In the former case compounds are liberated that otherwise might not be readily accessible to the environment and in the latter instance analytes of interest might be destroyed. In either case there is a possibility that important information may be lost.

Given the very heterogeneous nature of matrices and variety of problems found in environmental samples an empirical approach was required to develop filtration procedures for increasing the amount of natural waters that could be prepared on an Empore disk. A large number of filters were studied to determine compatibility both with the aqueous sample and any organic solvents that might be necessary for elution. Under these con-

straints only two classes of filter proved acceptable: glass fibre and nylaflo.

In the studies on preparation of 1–2-l samples, the filters were placed above and in direct contact with the Empore disk and the sample was passed through the combined filter/disk unit. This technique was termed on-line filtration. Both the chemico-physical nature and the pore size were important in determining the effectiveness at increasing the volume of sample that could be prepared in one h. Within the class of GMF filters, the pore size, as expected, had a significant effect (Fig. 1). Relative to the 2-μm GMF filter, one with 1 μm pore size afforded a two-fold decrease in the time to prepare 2 l of Hamilton Harbour water. Relative to the unfiltered case there was an eight-fold decrease in the time to prepare the 2 l of this sample when the sample was prepared via the 1-μm filter. The size of the pores was not the sole determinant of efficacy when different types of filters were used. Despite the four-fold differences in pores size, use of the 2-μm GMF and the 0.45-μm nylaflo filters gave comparable results for an on-line filtration (Fig. 1).

Increased efficiency with decreased pore size suggested that use of nylaflo filters with 0.2-μm pores might further increase the volume of waters that can be prepared in the 1–2-h time frame. Moreover, a possible source for occlusion of filter pores are microbes and since the 0.2-μm size is a sterilization filter, any microbiological matter would be trapped prior to passage through the Empore disk. A 47-mm nylaflo filter with 0.2-μm pore size was tested with the sample of Hamilton Harbour water collected in October 1991. Further reduction in pore size, rather than increasing flow rate, resulted in a filtration time of 130 min for 2 l of this sample. The long filtration time may be explained by the excessive blocking of the small pores of the pre-filter or by viscous drag through the small pores.

The substantially longer filtration times indicated that a different approach was required if the 0.2-μm pore nylaflo filters were to be used to remove particulates prior to the adsorption step on an Empore disk. An off-line filtration technique was studied in which the sample was first passed through the 0.2-μm nylaflo filter and the resulting filtrate was then passed through an Empore disk. The flow characteristics of this filtrate through the Empore disk were substantially improved. For instance, 2 l of unfiltered sample collected from Hamilton Harbour in October 1991 required 840 min to pass through the 47-mm Empore disk whereas the filtered water required only 70 min (Tables III and IV).

TABLE III

TIMES REQUIRED FOR UNFILTERED WATER TO FLOW THROUGH A 0.2-μm NYLAFLO FILTER

Diameter of 0.2-μm pore size nylaflo filter (mm)	Volume (l) of October sample of natural water filtered through nylaflo filter	Time required (min)
47	2	131
90	2	9
90	5	128

These data suggested that efficient application of the sterilization filter would be to use larger-diameter filters prior to the adsorption onto the 47-mm Empore disks. A smaller Empore disk was considered preferable as this would minimize the volume of solvent needed for elution of analytes. In order to test this technique a 2-l sample of Hamilton Harbour water collected October 1991 was filtered water through a 90-mm nylaflo filter with 0.2-μm pore size and this step was complete in only 9 min.

A doubling of the diameter of the filter should increase the flow-rate by only four-fold yet in this instance there is a 15-fold decrease in the time required to collect 2 l. It should be recognized that these studies were conducted with natural waters that contained a particulate load. In such instance

TABLE IV

TIMES REQUIRED FOR WATER INITIALLY FILTERED THROUGH NYLAFLO TO FLOW THROUGH AN EMPORE DISK

Diameter of empore disk (mm)	Volume of pre-filtered water (l) passed extracted on an Empore disk	Time required (min)
47	2	70
90	5	21

TABLE V

LARGE VOLUME FLOW THROUGH 90-mm DISK

Filter type and pore size	Filtration time (min) for 2 l	Filtration time (min) for 5 l	Filtration time (min) for 10 l	Filtration time (min) for 11 l
GMF 2 μm	5	27	90	ND
GMF 1 μm	6	21	56	ND
GMF 1 μm	7	23	63	75
GF/A 1.6 μm	9	17	33	ND

the flow-rate decreases exponentially with volume filtered [7] (Fig. 1 and Table V). Accordingly, whereas with a 47-mm filter, the flow-rate is markedly decreased with filtration of more than 1 l a similar decrease in flow-rates is not observed with a 90 mm filter until more than 5 l are collected (Table V). Filtration of 2 l through the 90-mm filter thus occurs in throughout region of the volume filtered *vs.* time curve where for this size filter the flow-rate is very fast and so a sixteen-, rather than four-fold decrease in filtration time is observed.

Again the filtrate from the 90-mm nylaflo filter with 0.2-μm pore size could be passed through a 47-mm Empore disk in 70 min; thus the total time for adsorption step for 2 l of sample was 79 min as opposed to 209 min if both the filter and Empore disk were 47 mm in diameter. Thus, if small pore sizes are required (*e.g.* to remove microbes) then acceptable sample preparation times can be achieved filtering the sample through a larger-diameter filter prior to extraction which could be done on a smaller-diameter Empore disk.

Use of either of the GMF or the nylaflo filters produced some losses. The GMF filters are thick and this resulted in an approximate 10% loss of sample due to wicking and resultant leakage through the sides [7]. This problem may have been due to a poor design of the holder where clamping pressure is applied on one side only and the opposite side is thus not sufficiently compressed. The nylaflo filters are thinner and not subject to the same losses. These filters, however, (but not those of the GMF group) retained analytes with higher molecular masses such as DDT and DDE and reduced the yields by approximately 20-30%.

These results suggest that the GMF and nylaflo filters could be used for different applications. For instance, the nylaflo class would be suitable for determination of total analyte (*e.g.* water soluble and particulate bound). In this case the filter (containing both particulates and some organics adsorbed on the nylaflo filter) and Empore disk (containing a substantial part of the organics dissolved in water) would be extracted together to recover total organics in water and sediment. The GMF filters (with better designed holder) would be suitable for a independent analysis of the compounds in the liquid and solid phases. In this case, the filter and the Empore disk would be separated and the two fractions independently extracted. The isolate from the filter would contain analyte initially bound to particulates and the isolate from the Empore disk would contain analytes that were initially dissolved in water.

In order to increase the amount of water collected the size of both the filtration and Empore disks was increased to 90 mm. With a 1-μm GMF filter on-line with the Empore disk, 10 l of sample collected from Hamilton Harbour in October 1991 could be passed through combined filter/Empore disk in 1 h (Table V). When a similar glass fibre filter, the 1.6-μm GF/A was used, the time for preparation of 10 l of the same water decreased to 33 min. By allowing the collection of 10 l use of the 90-mm disks can readily produce concentration factors of 10 000–20 000 fold. The data in Table V where two experiments on filtration through a 1-μm GMF filter again shows that filtration times are reproducible and also demonstrates the exponential decrease in flow-rates with volume collected.

Drying procedure for an Empore disk

In addition to decreasing the time for the adsorption step [7], it also proved simpler to dry and Em-

TABLE VI

SUCCESSIVE WASHES FOR RECOVERY OF INDIVIDUAL COMPONENTS: PERCENT OF TOTAL RECOVERY IN 30 min

Normalized to final recovery = (absolute recovery in fraction/final absolute recovery) × 100.

Component	Recovery in 10 min: absolute recovery (normalized to final recovery)	Recovery in 20 min: absolute recovery (normalized to final recovery)	Recovery in 30 min
Methoxychlor	77	96	100
Heptachlor	37 (76.3)	46 (94)	49 (100)
Mirex	32 (76.9)	39 (97)	41 (100)
Aldrin	28 (79)	35 (98)	36 (100)
Cummulative recoveries normalized to final recovery	(78)	(96.5)	(100)

pore disk than to dry a cartridge. Drying of the solid phase is required in all of SPSP techniques for two reasons: (i) a coating of water on the solid phase can reduce efficiency of the desorption for analytes adsorbed on the solid phase by water-immiscible solvents; (ii) whether by desorption or physical displacement water "co-elutes" from the solid phase with the eluting solvents which must be dried prior to further sample work-up or instrumental analysis. Drying solid phase in the column configuration requires filtering air through the SPSP phase at 100 ml/min to remove water from the solid phase [2]. Such a high flow-rate can cause losses of the more volatile constituents and in addition requires extra manipulation: first to link the gas lines to the cartridge, and then to detach the cartridge for subsequent isolation of analytes. The disk configuration, however, exposes a large area to the atmosphere and as a result the Empore disks can be dried by simply placing in a desiccator for 1 h.

Recoveries

Several recovery studies were performed by adding aliquots of the organochlorine spike to environmental water, tap water or distilled water and extracting using SPSP. Initial recoveries were high but with unacceptable variability; relative standard deviations were 32.4% (for spikes in the 0.5–5.0 ng/ml range), 30.9% (for spikes in the 2.5–25 ng range), 25.2% (for spikes in the 5–50 ng range) (data not shown). The reason for the unacceptable R.S.D.s stems from the loss of components in the concentration and evaporation step. By using a more controlled evaporation and using 0.5 ml of iso-octane as a keeper solvent, reproducibility became more acceptable with R.S.D.s ranging from 8.6 to 18.8% (Table VI) in the 100 ng/l concentration range.

The studies on recovery identified three classes of compounds: those recovered in high yield; those obtained in low yield due to breakthrough and those obtained in low yield for causes (as yet undetermined) other than breakthrough. Half of the compounds studied were recovered in yields of 70% or more (Table VII). One compound, 2,4-dichlorophenol, was recovered in low yield but this was due to breakthrough as shown by the fact that the filtrate contained the "missing" portion of the analyte. Three of the 12 compounds (heptachlor, mirex, aldrin) were recovered in yields below 50% but the reduced yield was not due to breakthrough. Less than 2% of these three analytes initially spiked into the water was recovered from a re-analysis of the water that had passed through the disk. Losses on glass-ware of these three compounds was considered and so the containers used to make up the spiked aqueous solutions were washed with methanol but no analytes were recovered in this wash.

It was also considered possible that the low re-

TABLE VII

PERCENT RECOVERIES OF INDIVIDUAL COMPONENTS FROM TAP OR DEIONIZED WATER

Methods: A = 10 min leech time with recoveries being determined by GC–ECD; B = 30 min leech time with recoveries being determined by GC–ECD; C = elute analytes with methanol and determine recoveries by scintillation counting.

Component	Yield (%)	R.S.D. (%)	No. of trials	Method
Dieldrin	94	18.8	5	A
p,p'-DDE[a]	74	17.5	5	A
Endrin	132	16.2	5	A
p,p'-TDE[b]	100	8.62	5	A
o,p'-DDT[c]	100	8.62	5	A
Methoxychlor	90	9.64	5	A
Methoxychlor	103	ND	1	B
Heptachlor	48.7	9.64	5	B
Mirex	40.5	3.01	4	B
Aldrin	36.6	6.5	5	B
2,4-D[d]	69.2	11.2	5	C
2,4-Dichlorophenol	36.1	6.34	5	C

[a] *p,p'*-Dichloro-2,2-bis(*p*-chlorophenyl)ethylene.
[b] Tetrachlorphenylethane.
[c] *o,p'*-1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane.
[d] 2,4-Dichlorophenoxyacetic acid.

covery of heptachlor, mirex and aldrin may have been due to insufficient time for elution of the analytes from the disk. The effect of elution time on recovery of analyte was therefore determined. A leeching technique was used to simplify control of the contact time between eluting solvent and solid phase. To this end the kinetics of the extractions were studied for four selected compounds (methoxychlor, heptachlor, aldrin, mirex) that had been adsorbed onto an Empore disk from tap water. It was found that an average of 78% of the total amount of analyte ultimately recovered was present in isolate after the first 10 min. An additional 18% were recovered by leeching the disk for another 10 min and only a 4% gain for the total 30-min leech (Table VII). This pattern of recovery was independent of whether the analyte was finally obtained in high or low yield. Similar results were obtained by leeching the disk in a large volume of diethyl ether and removing small aliquots at 10-min time intervals: 78% of the analyte ultimately recovered was present in the diethyl ether after the first 10 min of leeching and 94% was present after 20 min. However, while the second method requires less manipulation, the first method of leeching and decanting the eluant from the disk provides more consistent recoveries with R.S.D.s for the four separate analytes of less than 5%. These data suggested that losses did not result from insufficient extraction time. If low yields for compounds such as mirex were due to slow elution from the disk then the fraction recovered with longer extraction times would have been higher.

A 1-l volume of water (Hamilton Harbour) collected in July was extracted using a 47-mm Empore disk and the analytes were eluted from the solid phase by leeching with diethyl ether for 20 min. This provided sufficient amounts of analyte for detection of organic electrophores (Fig. 2). Several of these compounds could be identified on the basis of their retention times (min) in comparison to a spiking solution containing these components: penta- and hexachlorobenzene (7.26, 7.76), heptachlor (11.58), aldrin (13.03), γ-chlordane (16.07), α-chlordane (16.56), and endrin (18.71). Several components with retention times of 8.12, 21.42 and 25.69 min

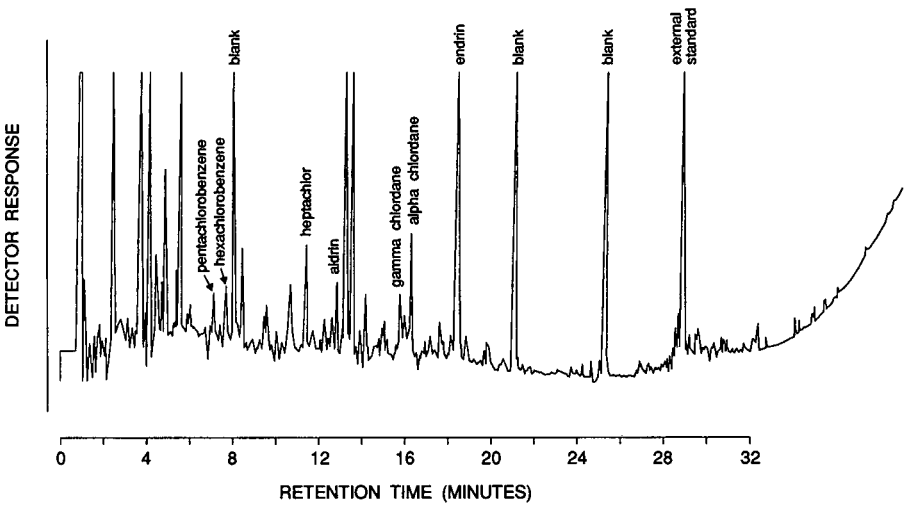


Fig. 2. Gas chromatographic trace with electron-capture detection of 1 l extract of Hamilton Harbour water on a 47-mm octylsilica Empore disk.

were also present in the extraction of 1 l of distilled or doubly distilled water demonstrating that these were contaminants from laboratory equipment or from the Empore disk. The remaining peaks could only be tentatively identified as organochlorines or phthalates on the basis of being detected by ECD.

Removal of non-specific interferences

When larger volumes of Hamilton Harbour water were extracted, elution of analytes with diethyl ether resulted in a co-elution of standard organochlorine analytes with highly pigmented material and materials that caused considerable NSI in the capillary GC–ECD trace (Fig. 3). It is not yet clear whether the pigmented and the NSI material are one and the same. An investigation of this problem demonstrated another possible advantage to using the Empore disk; the feasibility of selective elution.

The initial approach to separate NSI from analytes was use of semi-preparative normal phase col-

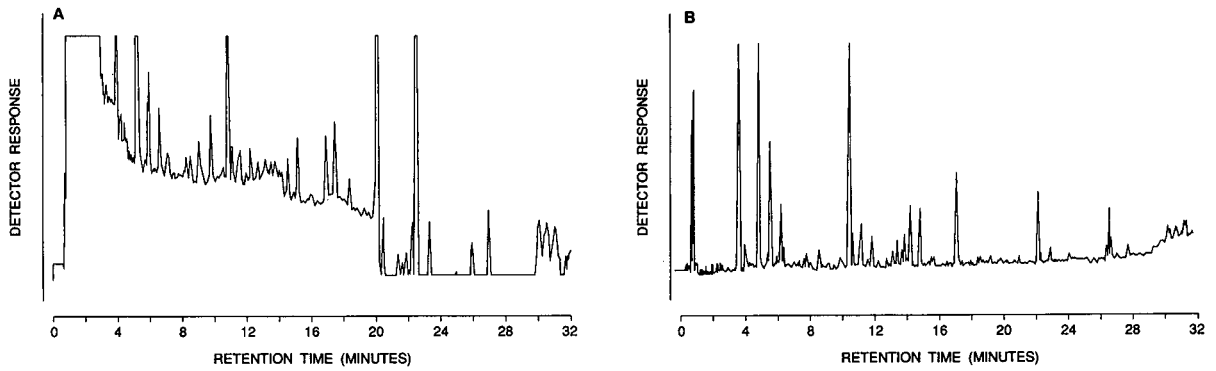


Fig. 3. (A) Capillary GC–ECD trace of a 10-l extract of Hamilton Harbour water (October 1991) using a 90-mm octylsilica Empore disk with a 1.0-μm GMF filter. Organics adsorbed on the disk were extracted with diethyl ether and concentrated with no further clean-up. (B) 10-l extract Hamilton Harbour water (October 1991) using a 90-mm octylsilica Empore disk with a 1.0-μm GMF filter. Organics adsorbed on the disk were extracted with diethyl ether and separated by elution from a Florisil column with diethyl ether–isooctane (5:95, v/v).

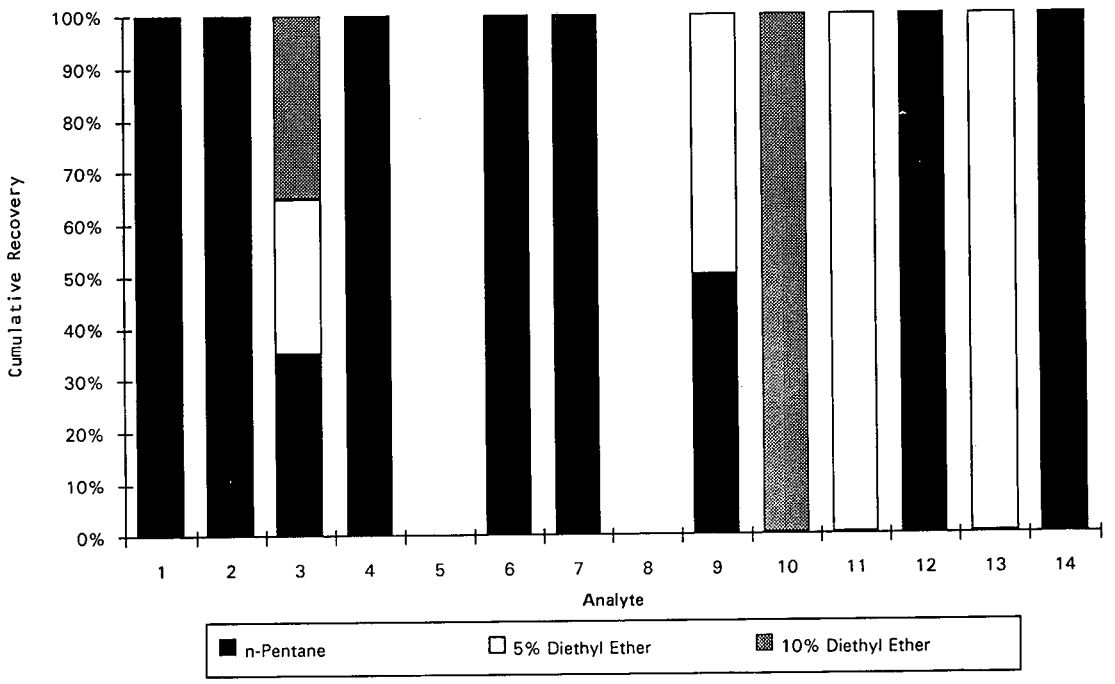


Fig. 4. Cumulative recoveries from a Florisil column by elution with *n*-pentane, 5 and 10% diethyl ether in *n*-pentane. Compounds: 1 = hexachlorocyclohexane; 2 = hexachlorobenzene; 3 = lindane; 4 = heptachlor; 5 = heptachlor epoxide; 6 = γ-chlordane; 7 = α-chlordane; 8 = dieldrin; 9 = *p,p'*-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE); 10 = endrin; 11 = β-endosulphan; 12 = tetrachlorphenylethane (*p,p'*-TDE) + *o,p'*-1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (o,p'-DDT); 13 = methoxychlor; 14 = mirex.

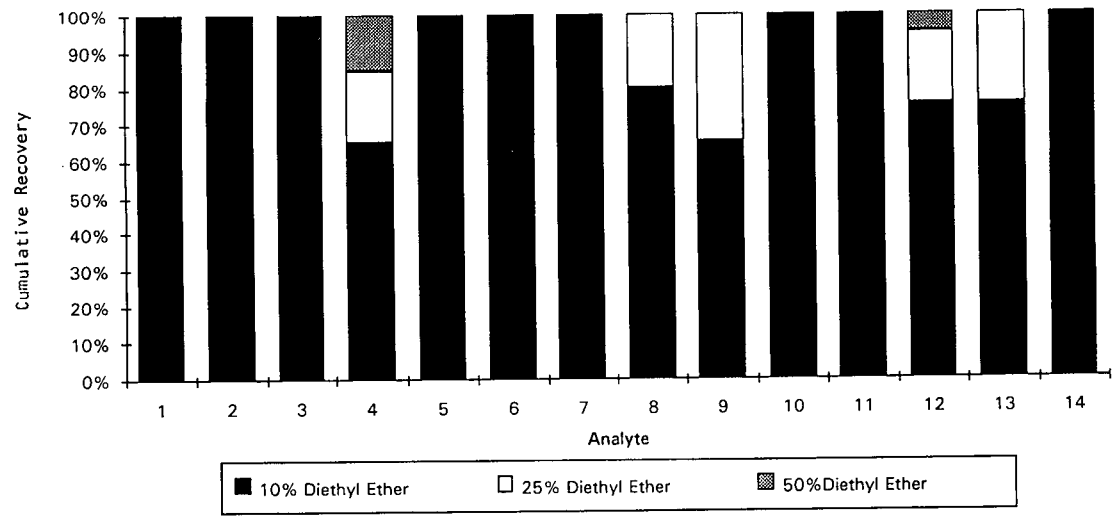


Fig. 5. Cumulative recoveries by leeching adsorbed analytes directly from an Empore disk with 10, 25 and 50% diethyl ether in *n*-pentane. Compounds: 1 = hexachlorocyclohexane; 2 = hexachlorobenzene; 3 = lindane; 4 = heptachlor; 5 = heptachlor epoxide; 6 = γ-chlordane; 7 = α-chlordane; 8 = dieldrin; 9 = *p,p'*-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE); 10 = endrin; 1 = β-endosulphan; 12 = tetrachlorophenylethane (*p,p'*-TDE) + *o,p'*-1,1,1 trichloro-2,2-bis(*p*-chlorophenyl)ethane (*o,p'*-DDT); 13 = methoxychlor; 14 = mirex.

umn chromatography. The organics were first eluted from the Empore disk and concentrated in isooctane or hexane. This isolate was transferred to a column of Florisil for separation. (This phase has retentivity intermediate between silica and alumina). Elution with 10% diethyl ether in pentane recovered 12 of the 14 target organochlorine analytes in varying yield (Fig. 4). In order to recover the additional two analytes elution with 25% diethyl ether in pentane and then with 50% diethyl ether in pentane was attempted but the NSI began to elute with the former solvent system and more NSI was recovered with the latter eluent. These data suggested that normal-phase chromatography was not completely effective in separating analytes from the coloured material and/or the NSI.

The second separation technique was based on selective elution from the disk. In this case solvent of differing polarities were used to elute the compounds directly from the disk. The data in Fig. 5 shows that leeching the Empore disk with 10% diethyl ether in pentane recovers all of the compounds but does not optimally recover each analyte. Nevertheless all compounds spiked into the sample are isolated in greater than 65% of the final yield of any given analyte. More important, 10% diethyl ether in pentane does not elute either the coloured material and/or the NSI. In contrast, while 25% diethyl ether in pentane does give a more complete recovery this solvent system also elutes a sufficient amount of pigment and/or NSI is eluted so as to compromise the capillary column GC–ECD analysis.

Comparison of liquid–liquid extraction and SPSP with an Empore disk

Liquid–liquid extraction and SPSP of large volumes of Hamilton Harbour water were compared using recovery of ambient compounds as the basis for comparison. Despite the fact that a direct comparison (*i.e.* comparison of recoveries of analytes from two aliquots of the same water sample of water prepared by the same technician on the same day) could not be made a substantial fraction (8/13) of ambient compounds were recovered in comparable yield (Table VIII). Three compounds were recovered in greater yield by liquid–liquid extraction but this may have been due to losses on storage. The higher recovery for heptachlor with liquid–liquid extraction may, however, may accurately reflect the fact that recoveries for this compound by SPSP were low. One compound was extracted in higher yield by SPSP possibly reflecting a more efficient extraction. These data suggest that SPSP with the Empore disk is a viable alternative to liquid–liquid extraction for the recovery of ambient organic analytes from natural waters.

TABLE VIII

COMPARISON OF RELATIVE RECOVERIES OF ANALYTES FROM NATURAL WATERS USING USING LIQUID–LIQUID EXTRACTION (LLE) OR SOLID-PHASE SAMPLE PREPARATION (SPSP)

Component	*R*1 for LLE	*R*1 for SPSP	Ratio of recoveries for LLE/SPSP
1	19.6	5.84	3.36
2	5.05	17.8	0.28
3	3.29	3.44	0.96
4	29.1	25.7	1.09
5 (hexachlorobenzene)	2.71	4.01	0.68
6	4.38	4.39	1.00
7	4.66	20.5	0.23
8	1.8	2.58	0.70
9	3.4	1.39	2.45
10 (heptachlor)	14.1	1.42	9.93
11 (aldrin)	5.69	5.80	0.98
12 (α-chlorodane)	2.84	3.39	0.84
13	4.34	3.77	1.15

CONCLUSIONS

Optimal use of the Empore disk for the analysis of natural waters requires that the problems of particulate matter and non-specific interferences be resolved. It proved possible to use filters set in-line or off-line with the Empore disks to remove particulate matter prior to extraction and thus relatively large volumes (10–11 l) of natural water could be treated. Non-specific interferences could also be separated by selective elution from the disk whereas semi-preparative chromatography on Florisil does not separate all analytes from NSI. Finally, the recoveries from the Empore disks are comparable to that obtained by liquid–liquid extraction using dichloromethane.

ACKNOWLEDGEMENTS

This work was supported by a grant from The Great Lakes University Research Fund.

REFERENCES

1 W. K. Wang and S. D. Huang, *J. Chromatogr.*, 483 (1989), 121–129.
2 W. E. Johnson, N. J. Fendinger and J. R. Plimmer, *Anal. Chem.*, 63 (1991) 1510–1513.
3 C. H. Marvin, I. D. Brindle, C. D. Hall and M. Chiba, *Anal. Chem.*, 62 (1990) 1495–1498.
4 M. W. F. Nielen, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 57 (1985) 806–810.
5 B. Crathorne, M. Fielding, C. P. Steel and W. D. Watts, *Environ. Sci. Technol.*, 18 (1984) 797–802.
6 D. R. Green and D. LePape, *Anal. Chem.*, 59 (1987) 699–703.
7 D. F. Hagen, C. G. Markell, G. A. Schmitt and D. D. Bleuins, *Anal. Chim. Acta*, 236 (1990) 157–164.
8 A. Kraut-Vass and J. Thoma, *J. Chromatogr.*, 538 (1991) 233–240.

Journal of Chromatography, 629 (1993) 55–59
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 672

Automated solid-phase extraction of herbicides from water for gas chromatographic–mass spectrometric analysis

M. T. Meyer, M. S. Mills and E. M. Thurman

Water Resources Division, US Geological Survey, 4821 Quail Crest Place, Lawrence, KS 66049 (USA)

ABSTRACT

An automated solid-phase extraction (SPE) method was developed for the pre-concentration of chloroacetanilide and triazine herbicides, and two triazine metabolites from 100-ml water samples. Breakthrough experiments for the C_{18} SPE cartridge show that the two triazine metabolites are not fully retained and that increasing flow-rate decreases their retention. Standard curve r^2 values of 0.998–1.000 for each compound were consistently obtained and a quantitation level of 0.05 μg/l was achieved for each compound tested. More than 10 000 surface and ground water samples have been analyzed by this method.

INTRODUCTION

The triazine and chloroacetanilide herbicides atrazine and alachlor are widely used pre-emergent herbicides in the midwestern USA. These herbicides have been reported as common contaminants in both surface and ground water [1–3]. Two dealkylated triazine metabolites, deisopropylatrazine and deethylatrazine, have also been detected [2,3]. As a result of this environmental problem several methods using gas chromatography–mass spectrometry (GC–MS) have been developed to analyze these herbicides [4–6]. However, regional reconnaissance and process studies usually require the analysis of large numbers of samples. Because of the extensive automation available for liquid chromatographic and GC–MS analysis the most time-consuming, expensive, and error-prone step then becomes the pre-concentration of the analytes of interest.

Solid-phase extraction (SPE) has been successfully used for the extraction of triazine and chloroacetanilide herbicides [7–9]; however, automated methods for the preparation of these frequently analyzed organic compounds are needed to reduce the costs of analysis, diminish sample handling and preparation errors, increase sample through-put, and increase safety. The most common techniques for the extraction of non-ionic, non-polar organic compounds from water are liquid–liquid extraction and SPE [10,11]. Of these techniques, SPE is best suited for automation because the small disposable cartridges containing small amounts of solid sorbent require little organic solvent and are readily fitted into a robotic system. Several other advantages of SPE over liquid–liquid extraction as a sample preparation technique also have been espoused [10,12,13].

Automation of SPE is a relatively new concept as commercial equipment for this purpose has become available within only the last 5–6 years. Furthermore, only within the last 4 years has an automated SPE workstation been available that was capable of processing more than three large samples of 100–1000 ml, which are necessary for environmental

Correspondence to: M. T. Meyer, Water Resources Division, US Geological Survey, 4821 Quail Crest Place, Lawrence, KS 66049, USA.

0021-9673/93/$06.00

analysis. This paper presents an automated method for the isolation of chloroacetanilide and triazine herbicides, and two polar metabolites of atrazine, deethylatrazine and deisopropylatrazine, from water using a Millilab 1A workstation (Waters Chromatography, Milford, MA, USA) with an on-line computer. Coupled with GC–MS–selected-ion monitoring (SIM) this is a robust method for the analysis of herbicides from water present at ng/l levels using only a sample of 100 ml.

EXPERIMENTAL

Apparatus

The Millilab 1A workstation with an on-line computer was used for the automated SPE of herbicides from water. Here we describe the pertinent set-up information for implementing this procedure. The two syringe pumps on the fluidics module were outfitted with a 5- and 1-ml syringe. Two multiple intake accessories (MIAs) were added to the 5-ml syringe on the fluidics module to increase the number of water samples that could be processed from 3 to 14. Also a distilled water reservoir, for rinsing the PTFE tubing and XYZ probe, was connected to one of the valves of an MIA. Solvent reservoirs of distilled water, ethyl acetate, and methanol, attached to the 1-ml syringe, were used as working solvents for elution of the SPE cartridges and the pipetting of reagents and spiked sample eluates. Custom-designed Plexiglas racks that could hold up to fourteen 125-ml bottles were used to organize water and quality assurance samples into sets. The bottle racks were keyed to fit into a custom-designed Plexiglas housing that was mounted in front of the fluidics module. PTFE sample lines from the MIAs were held securely in the sample bottles by pre-drilled holes in the top of the Plexiglas housing, through which the hoses were strung.

The transport module of the Millilab workstation contained an XYZ probe used for pipetting and dispensing reagents, and delivering the water sample to the SPE cartridge. Also, the transport module had a test-tube rack, and SPE cartridge rack, which also contained positions to which small reservoirs containing pipetting reagents can be placed. To one of the reagent positions a custom designed Plexiglas rack was attached to hold a 10-ml screw-top test-tube so that an internal standard could be pipetted into each of the sample eluates.

Reagents

Pesticide-grade methanol and ethyl acetate were obtained from Fisher Scientific (Springfield, NJ, USA). Ametryn, atrazine, prometon, prometryn, propazine, simazine, and terbutryn were obtained from Supelco (Bellefonte, PA, USA); alachlor, cyanazine, metolachlor, metribuzin, terbuthylazine (the surrogate, recovery standard), and [$^2H_{10}$]phenanthrene (the internal standard) were obtained from EPA Pesticide Chemical Repository (Research Triangle Park, NC, USA). Two triazine metabolites, deethylatrazine and deisopropylatrazine, were obtained from Ciba Geigy Agricultural Division (Greensboro, NC, USA). All of the standards obtained were greater than 97% pure. Concentrated stock and spiking solutions were prepared in methanol, except for [$^2H_{10}$]phenanthrene which was prepared in ethyl acetate. Distilled water was generated by purification through activated charcoal filtration and deionization with a high-purity, mixed-bed resin, followed by another activated charcoal filtration step and finally distillation in a Wheaton Autostill-5 (Millville, NJ, USA). Sep-Pak plus cartridges, containing 360 mg of 40-μm C_{18} bonded silica packing, were obtained from Waters (Milford, MA, USA).

Extraction procedure

Surface and ground water samples collected for analysis were filtered through 0.7-μm glass-fiber filters (Geotech, Denver, CO, USA), then refrigerated in 125-ml glass bottles. Ten samples were placed into custom-made Plexiglas racks. Two distilled water solutions fortified with a herbicide mix and two blanks were then added to the sample racks for quality control. The concentration of the fortified distilled water solutions ranged from 0.05 to 5.0 μg/l. Then 100 μl of a surrogate standard, terbuthylazine (1.34 ng/μl), were added to each bottle.

The SPE cartridge was conditioned by passing 2 ml of methanol, 6 ml of ethyl acetate, 2 ml of methanol, and 2 ml of distilled water. Reagents were pipetted through the SPE cartridge from the reagent reservoirs on the transport module. The PTFE sample lines were primed with 23 ml of water sample prior to pumping the remaining 100 ml of sample through the SPE cartridge at a flow-rate of 20 ml/min. The probe was then rinsed with distilled water and filtered compressed air then was passed

through the SPE cartridge for 1 min to remove as much water sample trapped in the cartridge as possible. The SPE cartridge was eluted with 2.5 ml of ethyl acetate into a centrifuge tube. Each sample in the set was sequentially prepared in the same way. An internal standard, [$^2H_{10}$]phenanthrene (500 μl, 0.2 ng/μl), was then added to each of the sample eluates in a batch spiking procedure. The Millilab mixed the sample eluate, then pipetted the ethyl acetate into a clean centrifuge tube to separate it from the residual water in the SPE cartridge, which co-eluted with the ethyl acetate. The interior and exterior of the probe were then washed with ethyl acetate after each sample was mixed and pipetted. This cycle was repeated until all the samples were mixed and separated. The eluates were removed from the Millilab, reduced to a volume of approximately 100 μl using a Zymark Turbovap LV evaporator (Hopkinton, MA, USA), and transferred into 200-μl glass-lined polystyrene vials for analysis by GC–MS.

GC–MS analysis

. Sample eluates were analyzed using a Hewlett-Packard Model 5890 gas chromatograph and a 5970B mass-selective detector (Palo Alto, CA, USA). Operating conditions were as follows: a direct capillary interface at 280°C, ionization voltage 70 eV, ion source temperature 280°C, electron multiplier 400 V above autotune, tuned daily with perfluorotributylamine. The filament and electron multiplier were turned on 15 min into each sample run. Twenty-nine ions divided into four acquisition groups were monitored during each sample run. The area of the base-peak ion for each compound was divided by the single 188 ion-peak of the [$^2H_{10}$]phenanthrene and the 214 ion-peak of terbuthylazine for quantification. Compound confirmation was based upon the presence of the molecular ion, and one to two confirming ions (with area counts ±20%), and a retention time match of ±0.2% relative to [$^2H_{10}$]phenanthrene.

Samples were injected in the splitless mode into the gas chromatograph. The injector temperature was 280°C. Herbicides were separated on a 12 m × 0.2 mm I.D., HP-1 fused-silica capillary column with a film thickness of 0.33 μm (Hewlett-Packard). The helium carrier gas flow-rate was 1 ml/min with a head pressure of 35 kPa. The column temperature was held at 60°C for 1 min and programmed to ramp at 6°C/min to 250°C.

RESULTS AND DISCUSSION

Table I shows that only the two triazine metabolites were detected in the breakthrough determinations. As the flow-rate was increased from 20 to 60 ml/min the breakthrough of deisopropylatrazine increased from 35 to 40% and deethylatrazine from 5 to 10%. The standard deviations of the mean breakthrough for some adjacent flow-rates overlap for both compounds. Thus, it is not certain that there is discernible difference in breakthrough by increasing the flow-rate from 20 to 30 ml/min. However, there is a trend of increased breakthrough with increasing flow-rate. Furthermore, there is a distinguishable difference in breakthrough from 20 to 60 ml/min for both compounds. These data indicate that for compounds with low sorption capacity precise control of the flow-rates is necessary to ensure consistent quantitative results.

Deisopropylatrazine has less sorption capacity than deethylatrazine because it has one less carbon group in the alkyl sidechain and can undergo less hydrophobic interactions with the C_{18} resin. Break-

TABLE I

PERCENT BREAKTHROUGH FOR SEP-PAK C_{18} CARTRIDGE FOR 13 HERBICIDES AS A FUNCTION OF FLOW-RATE

Using 100-ml sample, 1 μg/l concentration for each compound, passed through two cartridges in tandem, cartridges eluted separately using Millilab procedure and analyzed by GC–MS. Breakthrough based on an external standard curve for each compound. Procedure performed for each flow-rate in duplicate.

Flow-rate (ml/min)	% mean breakthrough ± S.D.[a]	
	Deisopropylatrazine	Deethylatrazine
20	35 ± 1.9	5 ± 0.6
30	37 ± 2.5	7 ± 0.7
40	37 ± 1.1	9 ± 0.9
60	40 ± 0.3	10 ± 0.2

[a] Breakthrough not detected for the other 11 herbicides listed in the Reagents section.

through volumes for the triazine and acetanilide herbicides on C_{18} at a flow-rate of 4 ml/min have previously been reported [6]. The results from that study, with 10 and 100% breakthrough for deisopropylatrazine occurring after 75 and 225 ml of sample were passed through a C_{18} cartridge, are in good agreement with those presented in Table I.

The variation of the recovery ratio of terbuthylazine to [$^2H_{10}$]phenanthrene was calculated for 11 historical standard curves. The ratio was calculated by dividing the area of the 214 ion of terbuthylazine by the 188 ion of [$^2H_{10}$]phenanthrene. The relative standard deviation (R.S.D.) for the average ratio calculated from each standard curve varies from ±2 to 6% and the average R.S.D. is 4%. The deviation of the terbuthylazine to [$^2H_{10}$]phenanthrene ratio for the majority of samples measured was, in general, within ±10% of the ratio calculated from the standard curve being used. However, the percent deviation for most of the samples was only 3 to 7% greater than the R.S.D. for the recovery ratios calculated from the standard curves. Most of the deviations greater than ±10% are due to either a spiking error, usually the terbuthylazine, or because the full volume of sample was not pumped through the cartridge. Thus, most of the variation in this method is not from extraction efficiency from complex matrices but is from spiking error, or instrument malfunction.

For each compound r^2 values from 11 standard curves were between 0.998 and 1.000. The high r^2 values, obtained using both the [$^2H_{10}$]phenanthrene and the terbuthylazine as quantification standards, shows that a high degree of reproducible precision has been maintained with this automated method. Furthermore, herbicide concentrations calculated by both standards usually agree within ±10%. Because the 5.0 μg/l standards exerts a lot of leverage on these standard curves it is important to maintain r^2 values of 0.997 or greater to have good control over the 0.05 to 0.2 μg/l range in the standard curve.

Quantitation levels of 0.05 μg/l are achieved for each of the 13 herbicides. However, deisopropylatrazine and cyanazine give significantly less response than the other compounds. A chromatogram of our 13-compound mix published in a previous study [6] illustrates this point. For deisopropylatrazine the response is diminished because 35 to 40% of the compound is not sorbed to the SPE cartridge. Furthermore, cyanazine and deisopropylatrazine are susceptible to losses in the injection port as the injection sleeve becomes dirty [6], which raises the quantitation levels if the injection sleeve is not regularly replaced. The fortified distilled water solutions run with each set were used to detect deterioration in analyte response. When the slope of the standard curve was adjusted by more than 15% to compensate for this loss the injection sleeve was replaced. Finally, analysis of duplicate samples and fortified distilled water solutions by independent laboratories were used as an independent check for method accuracy. In general, the results from the inter laboratory comparisons agree within 10 to 20% of the results obtained from our laboratory.

CONCLUSIONS

The breakthrough experiments show that the C_{18} SPE cartridge has limited sorption capacity for deisopropylatrazine and deethylatrazine and that the sorption capacity for these compounds is reduced with increasing flow-rates. Therefore, for accurate quantitative analysis of compounds with limited sorption capacity, such as deisopropylatrazine, precise control of flow-rate is necessary. The recovery, and standard curve data along with the analysis of thousands of fortified distilled water solutions and several hundred duplicate samples show that the precision (% relative standard deviation) for this method is ±10% for each compound. Furthermore, inter laboratory comparison studies of fortified distilled water solutions, and duplicate samples show that this method is accurate. Also this automated SPE method has reduced the amount of man-hours required for extraction by more than 70%, increased precision by 5%, increased sample through-put by 200% using two Millilab workstations, and reduced significantly technician exposure to solvent fumes. Finally, automated SPE coupled with GC–MS–SIM is a robust and reliable method for the routine detection of herbicides in the sub-ppb levels using only a 100-ml water sample.

ACKNOWLEDGEMENT

We appreciate the financial support of the Surface-Water and Ground-Water Toxics Program of the US Geological Survey, and the Productivity En-

hancemant Funding Program, US Department of the Interior. Brand names are for identification purposes only and do not imply endorsement by the US Geological Survey.

REFERENCES

1 R. A. Leonard, in R. Grover (Editor), *Environmental Chemistry of Herbicides*, CRC Press, Boca Raton, FL, 1988, Ch. 3, p. 45.
2 E. M. Thurman, D. A. Goolsby, M. T. Meyer and D. W. Koplin, *Environ. Sci. Technol.*, 25 (1991) 1794.
3 W. E. Periera and C. E. Rostad, *Environ. Sci. Technol.*, 24 (1990) 1400.
4 M. Popl, Z. Voznakova, V. Tatar and J. Strnadova, *J. Chromatogr. Sci.*, 21 (1983) 39.
5 I. G. Ferris and B. M. Haigh, *J. Chromatogr. Sci.*, 25 (1987) 170.
6 E. M.Thurman, M. Meyer, M. Pomes, C. A. Perry and A. P. Schwab, *Anal. Chem.*, 62 (1990) 2043.
7 M. W. Brooks, J. Jenkins, M. Jimenez, T. Quinn and J. M. Clark, *Analyst*, 114 (1989) 405.
8 R. G. Nash, *J. Assoc. Off. Anal. Chem.*, 72 (1990) 487.
9 M. A. Ahel, K. M. Evans, T. W. Fileman and R. F. C. Mantoura, in *Preprints of Papers, 203rd ACS National Meeting Division of Environmental Chemistry*, 32 (1992) 407.
10 G. A. Junk, in *Preprints of Papers, 203rd ACS National Meeting Division of Environmental Chemistry*, 32 (1992) 309.
11 K. C. Van Horne, in *Preprints of Papers, 203rd ACS National Meeting Division of Environmental Chemistry*, 32 (1992) 347.
12 K. G. Furton, E. Jolly and J. Rein, in *Preprints of Papers, 203rd ACS National Meeting Division of Environmental Chemistry*, 32 (1992) 332.
13 R. E. Majors, in *Preprints of Papers, 203rd ACS National Meeting Division of Environmental Chemistry*, 32 (1992) 336.

Journal of Chromatography, 629 (1993) 61–65
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 689

Optimization of sample application conditions for solid-phase extraction columns

Chang-Yuan L. Hsu and Rodney R. Walters

Drug Metabolism Research, The Upjohn Company, Kalamazoo, MI 49001 (USA)

ABSTRACT

When extraction recoveries during solid-phase extraction are affected by the volume or type of matrix applied to the column, the assay is not very robust. Using as examples the extraction of four basic drugs from biological matrices using cyano and octadecyl solid-phase extraction columns, it is shown that the recovery from 1 ml of plasma can be good while the recovery from water or diluted plasma is poor. Control of sample pH was found to increase recoveries from the cyano column by improving adsorption during sample application. Addition of detergent was found to enhance recoveries from the octadecyl column by moderating retention and allowing the drugs to be eluted more easily.

INTRODUCTION

Although solid-phase extraction (SPE) is widely used for the cleanup of biological samples prior to chromatographic analysis [1,2], developing rugged methods based on solid-phase extraction is still somewhat of an art. One problem that we have frequently encountered is a biofluid volume and/or type dependent recovery of drug and internal standard. There are three potential causes of poor recoveries: incomplete adsorption of drug to the SPE stationary phase during sample application with the 'lost' drug thus eluting in the initial flow-through of the column; premature elution during column-washing; or incomplete elution during the final elution step.

Experimentally determined differences in recoveries of drug from plasma, urine, or water must be due to chemical or physical differences between these matrices which affect either the properties of the solute or the stationary phase. The most likely factors causing matrix-dependent recoveries are pH, ionic strength, and surface tension. This presentation will describe the results of experiments aimed at identifying the crucial variables for assay methods for two basic compounds extracted using cyano SPE columns and two other basic compounds extracted using C_{18} SPE columns, and show how the assays could be made more rugged with respect to recovery from water, plasma, and urine samples of various volumes.

Correspondence to: R. R. Walters, Drug Metabolism Research, The Upjohn Company, Kalamazoo, MI 49001, USA.

EXPERIMENTAL

Materials

The compounds tested for recovery, U-77567, U-88055, U-70226, and U-74747, were obtained from Upjohn (Kalamazoo, MI, USA). Sequanal quality trifluoracetic acid (TFA) was from Pierce (Rockford, IL, USA). Other chemicals were reagent grade and solvents were of HPLC or UV grade.

Apparatus

Solid-phase extraction was performed using Bond-Elut C_{18} and Cyano SPE columns containing 100 mg of stationary phase and a Vac-Elut solid-phase extraction manifold (Analytichem, Harbor City, CA, USA). The HPLC system used for the

measurement of U-77567 and U-88055 recoveries consisted of a Beckman 110B solvent delivery module set at a flow-rate of 1 ml/min, a Perkin-Elmer ISS-100 autosampler with a temperature-controlled sample tray thermostatted to 2°C, a Zorbax RX C_8 analytical column (5 μm, 250 × 4.6 mm I.D.) with a guard column (12.5 × 4.0 mm I.D.), and a Waters 470 fluorescence detector with an excitation wavelength of 275 nm and an emission wavelength of 310 nm. Samples were injected every 15 min and eluted isocratically with a mobile phase consisting of acetonitrile–0.01 *M* sodium acetate, pH 4.0 (10:90, v/v). The HPLC system used for the assay of U-70226 and U-74747 was a complex column-switching reversed-phase system which will be described separately [3].

Procedure

A 50-μl volume of a 200-ng/ml aqueous solution of U-77567 was mixed with 50 μl of a 300-ng/ml aqueous solution of U-88055 and various volumes of buffer, detergent, plasma (rat), urine (rat), etc. as shown in Table I. Additional water was added to bring the volume of each sample to at least 1 ml. The mixtures were applied to Bond-Elut Cyano SPE columns which had been preconditioned with 1 ml each of methanol and water. Following sample application, the columns were washed with 2 ml of water, dried for 3 min by sucking air through the columns using house vacuum (*ca.* 70 kPa), washed with 300 μl of hexane, and eluted by gravity with 300 μl of methanol containing 0.1% TFA. The last volume was forced out using pressurized nitrogen. Following evaporation and reconstitution, the recoveries were measured by reversed-phase HPLC against a non-extracted solution of the compounds.

A 10-μl volume of an acetonitrile solution containing 1100 ng/ml of U-70226 and 1500 ng/ml of U-74747 was mixed, in duplicates, with various solutions (including human plasma and urine) as shown in Table II, then applied to Bond-Elut C_{18} SPE columns which had been preconditioned with 1 ml each of methanol and water. Following sample application, the columns were washed with 1 ml of acetonitrile–methanol–water (25:25:50), dried for 3 min by sucking air through the columns using house vacuum (*ca.* 70 kPa), washed with 300 μl of hexane, dried again in the same manner for 10 min, and eluted by gravity with 500 μl of acetone–acetonitrile–triethylamine (TEA) (50:50:0.2). The last volume was forced out of the column using pressurized nitrogen. After evaporation, the samples were

TABLE I

RECOVERIES OF U-77567 AND U-88055 FROM VARIOUS MATRICES AFTER EXTRACTION USING CYANO SPE COLUMNS

Treatment		U-77567 recovery (%)	U-88055 recovery (%)
Water	1 ml	2	2
Plasma	0.01 ml	29	29
	0.1 ml	95	99
	1 ml	100	106
Urine	0.01 ml	16	16
	0.1 ml	96	99
	1 ml	103	109
0.1 *M* pH 5 buffer	0.1 ml	57	53
	1 ml	70	81
0.1 *M* pH 7 buffer	0.1 ml	101	105
	1 ml	101	105
0.1 *M* pH 9 buffer	0.1 ml	96	99
	1 ml	99	104
Saline	0.1 ml	34	30
	1 ml	37	43
5% Dextrose	0.1 ml	3	2
	1 ml	3	2
0.04% Tween 80	1 ml	17	2
0.04% Triton X-100	1 ml	4	4

TABLE II

RECOVERIES OF U-70226 AND U-74747 FROM VARIOUS MATRICES AFTER EXTRACTION USING C_{18} SPE COLUMNS

Treatment		U-70226 Recovery (% ± S.D.) (n = 2)	U-74747 Recovery (% ± S.D.) (n = 2)
Plasma + 0.05 *M* pH 7 buffer–0.1% TEA	1 ml each	106 ± 1	104 ± 3
Urine + 0.05 *M* pH 7 buffer–0.1% TEA	0.1 ml urine, 1 ml buffer	76 ± 13	51 ± 10
Water + 0.05 *M* pH 7 buffer–0.1% TEA	1 ml each	78 ± 0	54 ± 3
Water	1 ml	54 ± 1	33 ± 1
Plasma	1 ml	71 ± 3	68 ± 3
Plasma	0.1 ml + 0.9 ml water	67 ± 2	59 ± 1
Urine	1 ml	66 ± 1	46 ± 1
Urine	0.1 ml + 0.9 ml water	59 ± 4	40 ± 5
0.1 *M* pH 5 buffer	1 ml	64 ± 0	48 ± 1
0.1 *M* pH 7 buffer	1 ml	55 ± 2	41 ± 2
0.1 *M* pH 9 buffer	1 ml	79 ± 1	60 ± 0
Saline	1 ml	73 ± 3	53 ± 1
Saline–0.1% TEA	1 ml	29 ± 6	42 ± 7
5% Dextrose	1 ml	57 ± 6	36 ± 6
0.04% Tween 80	1 ml	96 ± 3	104 ± 1
0.04% Triton X-100	1 ml	94 ± 1	95 ± 2
0.04% Triton X-100–0.1% TEA	1 ml	57 ± 2	97 ± 2

derivatized with 1-naphthyl isocyanate [4] and assayed by reversed-phase column-switching HPLC [3]. Recoveries were measured against a derivatized, non-extracted solution of the compounds.

The buffers used to dilute the samples in Tables I and II were all sodium phosphate buffers of the indicated pH and molarity. The pH of each buffer containing TEA was adjusted after addition of TEA.

RESULTS AND DISCUSSION

U-77567 (Fig. 1) is a basic compound assayed in biofluids using solid-phase extraction (cyano column) followed by reversed-phase HPLC. Recoveries of drug and internal standard (U-88055, Fig. 1) were high for 0.1–1 ml samples of both plasma and urine but low if 10 μl of plasma or urine were diluted to 1 ml with water prior to extraction due to their high drug content (same total amount of drug in all cases). It was suspected that this biofluid volume-dependent extraction recovery problem was caused by incomplete adsorption of drug to the cyano phase during sample application. Therefore, several experimental factors, such as pH, ionic strength, and the use of detergents, were tested for their effects on drug recovery.

The data from the experiments with U-77567 and U-88055 are shown in Table I. The results clearly

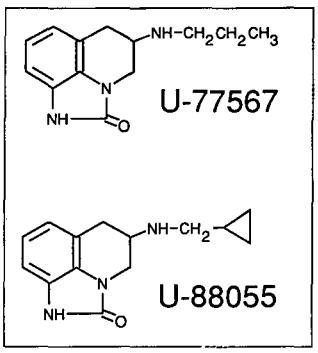


Fig. 1. Structures of U-77567 and U-88055, which were solid-phase extracted using a cyano column.

show the diminished recoveries when water or small plasma or urine volumes were extracted. This was obviously a pH effect, since addition of pH 7 or 9 buffers to the samples resulted in high recoveries, whereas ionic strength changes and addition of detergents did not alter the recoveries substantially. Since the pK_a values of these compounds are approximately 8, it can be concluded that the recovery problem was a simple equilibrium problem, with retention being too weak when the pH was not optimal. Apparently large plasma and urine samples provided their own buffering capacity, while samples diluted with water did not have sufficient buffering capacity to control the pH of the column.

U-70226 (Fig. 2), another basic drug, and its internal standard (U-74747, Fig. 2) were assayed by reversed-phase HPLC after solid-phase extraction on an octadecyl phase and derivatization of the alcohol moiety with 1-naphthyl isocyanate [3,4]. It was discovered that the recovery of drug from plasma samples was greater than from water and from other biofluids including urine, tissue homogenates, saliva and microsome samples. This problem was initially believed to be a result of variable adsorption during application to the SPE column. An experiment was conducted in which the compounds were applied to the SPE column in 1 ml of water. The flow-through was collected, mixed with 1 ml of plasma and 1 ml of pH 7 buffer–0.1% TEA, and reextracted (the latter conditions were known to result in high recoveries, see Table II). However, the compounds were not found in the initial flow-through, indicating that the problem was not one of poor initial adsorption.

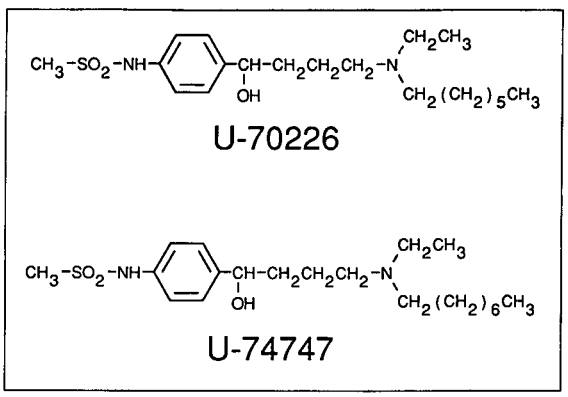


Fig. 2. Structures of U-70226 and U-74747, which were solid-phase extracted using an octadecyl column.

The original SPE column was then eluted three times with 0.5 ml volumes of the acetone–acetonitrile–TEA elution solvent. The recoveries of U-70226 and U-74747 were 67 ± 7% and 48 ± 7%, respectively, in the first fraction, 14 ± 3% and 11 ± 2% in the second fraction, and none in the third fraction. The total recoveries of 81% and 59%, respectively, after elution along with the lack of drug in the application flow-through suggest that these compounds are tightly adsorbed to the SPE stationary phase and are incompletely eluted by the elution solvent. In contrast, when the sample was applied in plasma, quantitative recoveries were achieved in the first 0.5 ml elution volume (Table II).

This problem was further examined by applying the compounds in a variety of solutions (Table II). Recoveries were lower when the drugs were mixed with water than when mixed with urine, and lower in urine than in plasma. Smaller volumes of plasma or urine (with additional water added) also resulted in lower recoveries. Recoveries were not improved by mixing the drugs with buffers of various pH values, saline, or dextrose. Addition of detergents, either Triton X-100 or Tween 80, did result in nearly quantitative recoveries. Addition of a pH 7 buffer containing TEA to the drugs in urine, water or plasma also improved recoveries, with recoveries being quantitative in the latter case. However, unbuffered TEA solutions (in saline or detergent) resulted in lower recoveries.

These data suggest that the C_{18} SPE phase contains strong adsorption sites. Some of these may be strong hydrophobic sites, others may be polar sites from exposed silanol groups. Addition of plasma, detergents, or buffered TEA during sample application each help to mask some of these sites, resulting in higher elution recoveries of drug. The highest recoveries were obtained by applying the samples in plasma and buffered TEA or in a detergent solution.

CONCLUSIONS

In the two examples given, matrix type and volume-dependent recoveries were found to be improved by addition of various reagents to the sam-

ple solution applied to the SPE columns. Testing the effects of buffers, detergents, etc. on solid-phase extraction recoveries during method development is useful in designing more rugged assays. If critical parameters can be identified, it may be possible to design assays such that any volume or type of matrix can be assayed without modification of the assay procedure.

REFERENCES

1 *Applications Bibliography Sample Preparation Products*, Varian Sample Preparation Products, Harbor City, CA 1991.
2 P.D. MacDonald (Editor), *Waters Sep-Pak Cartridge Application Bibliography*, Waters, Milford, MA, 5th ed., 1991.
3 C.L. Hsu and R.R. Walters, in preparation.
4 C.L. Hsu and R.R. Walters, *J. Chromatogr.*, 550 (1991) 621.

Journal of Chromatography, 629 (1993) 67–74
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 606

Alternatives to methanol–water elution of solid-phase extraction columns for the fractionation of high log K_{ow} organic compounds in aqueous environmental samples

Elizabeth Durhan

Environmental Research Laboratory–Duluth, US Environmental Protection Agency, 6201 Congdon Boulevard, Duluth, MN 55804 (USA)

Marta Lukasewycz and Steven Baker

AScI Corporation, 112 East Second Street, Duluth, MN 55805 (USA)

ABSTRACT

A toxicity-directed method for fractionating non-polar organic toxicants using solid-phase extraction (SPE) is described in phase II of EPA's "Methods for aquatic toxicity identification evaluations". This method has been used very successfully to extract and fractionate acutely and chronically toxic complex effluents and ambient waters. However, when fractionating samples that contain very hydrophobic (high log K_{ow}) toxicants the methanol–water elution sequence requires modification for optimum results. An elution modification has been made to the phase II SPE fractionation method for use with aqueous samples which contain such compounds (*e.g.* sediment pore water). The modified elution and fractionation method has been found to be effective for the separation and isolation of a mixture of compounds with log K_{ow} values ranging from 2.5 to 7 from aqueous solution and for toxicants from a sediment pore water sample.

INTRODUCTION

It is a difficult task to identify the contaminants which cause toxicity in complex environmental samples. A chemical screening method such as priority pollutant analysis may fail to identify the toxicants because the sample may contain thousands of compounds and there is no assurance that those measured are responsible for the toxicity. One approach for separating toxic from non-toxic components is the use of toxicity guided fractionation [1–4]. This approach uses organism response as the "toxicity detector". The US Environmental Protection Agency (USEPA) has developed a method to identify acutely toxic compounds in aqueous environmental samples using freshwater fish or invertebrates as the toxicity detector. This method, entitled "Methods for aquatic toxicity identification evaluations" (TIE), uses a three phase approach: phase I, toxicity characterization [5]; phase II, toxicity identification [6]; and phase III, toxicant confirmation [7]. These procedures have been used successfully to identify toxicants in wastewater effluents [1,8,9], ambient water [10], and sediment pore water [11,12]. Typical toxicants identified were cationic metals, ammonia, chlorine, pesticides, and non-polar organics. The identification procedure for non-polar organic toxicants described in phase II of the TIE methods relies on reversed-phase chromatography to separate toxic from non-toxic sample components [13–15]. The reversed-phase chromatography is achieved with C_{18} solid-phase

Correspondence to: Elizabeth Durhan, Environmental Research Laboratory–Duluth, US Environmental Protection Agency, 6201 Congdon Boulevard, Duluth, MN 55804, USA.

extraction (SPE) and a methanol–water solvent system. This method has been used very successfully to fractionate and isolate non-polar organic toxicants from complex effluents [1,2,8]. However, other aqueous samples, such as sediment pore water, often contain toxicants not typically found in effluents, which are not effectively fractionated by the phase II method. For example, compounds such as polyaromatic hydrocarbons, polychlorinated biphenyls and long chain aliphatic hydrocarbons have been implicated in the toxicity of sediment pore waters [16]. These compounds are highly hydrophobic, as is evidenced by their high log K_{ow} values. Such hydrophobic compounds are not well fractionated by the phase II method. To overcome this limitation a study was conducted which evaluated alternative solvent systems and alternate sorbents, to achieve optimum toxicant separation and recovery over a broad range of log K_{ow} values. One major limitation of the solvent system choice is that the fractionation method is toxicity directed, therefore all SPE fractions must be tested with aquatic organisms to track the toxicity. Methanol is one of the very few solvents that these organisms can tolerate at the percentage levels used in the aqueous exposure tests. When solvents were evaluated for their efficacy in eluting highly hydrophobic compounds we were left with very few choices that met our toxicity testing requirements. Problems such as high toxicity, the formation of azeotropes or immiscibility with methanol eliminated the use of solvents such as acetonitrile, acetone, diethyl ether and hexane. Methylene chloride was selected because of its solvent strength and because it can be removed from a methanol–methylene chloride mixture leaving a methanol fraction that can be tested for toxicity.

EXPERIMENTAL

Instrumentation

GC–MS. A Hewlett-Packard (Palo Alto, CA, USA) Model 5970B mass selective detector with a Model 5980A gas chromatograph, Model 7673A automatic liquid sampler and HP-UX series Chemstation.

HPLC. A Hewlett-Packard Model 1090 liquid chromatograph with a diode array detector and an Isco (Lincoln, NE, USA) Foxy fraction collector were used for HPLC analyses.

Materials

SPE. The two SPE sorbents evaluated were octadecylsiloxane (C_{18}) and octylsiloxane (C_8) bonded to silica. The SPE columns were obtained from J.T.Baker (Phillipsburg, NJ, USA) and contained 1 g of sorbent.

XAD. Two resins were evaluated: prepurified XAD-4 and XAD-7 obtained from Alltech Associates, Inc. (Deerfield, IL, USA).

High-purity water was obtained from a SuperQ system of Millipore (Bedford, MA, USA). High purity Burdick & Jackson (Muskegon, MI, USA) methanol, methylene chloride and high-purity water were used for all fractionations.

A test mixture containing compounds with a range of log K_{ow} values of 2.5 to 7.0 was used to evaluate chromatographic separations of the SPE and XAD resins. Chemicals in the test mixture and their estimated log K_{ow} are listed in Table I. The stock solutions of the test mixture compounds were prepared in acetone, and the sorbent loading solution contained approximately 10 μg/l of each compound in high-purity water with 5% methanol.

Methods

SPE loading and fractionation. A 1-g SPE column was conditioned by pumping 25 ml methanol through the column, followed by 25 ml high-purity water. A 950-ml volume of loading solution (unfiltered) or sediment pore water (filtered through a 0.45-μm nylon filter) was then passed through the column at a rate of 5 ml/min. The post-column so-

TABLE I

TEST MIXTURE COMPOSITION

Compound	Abbreviation	Estimated Log K_{ow}[a]
Diethyl phthalate	DEP	2.57
Naphthalene	NAPH	3.32
Phenanthrene	PHEN	4.49
Chrysene	CHRY	5.66
Hexachlorobenzene	HCB	6.42
p,p'-DDE	DDE	6.94

[a] Calculated using the CLOGP program of A. J. Leo, Pomona College, Claremont, CA, Medchem Project, CLOG-3.3 computer program, 1985.

TABLE II

COMPOSITION OF 11 RECOMMENDED FRACTIONS IN MODIFIED ELUTION SCHEME

Fraction	Composition of eluting solvents (%, v/v)		
	Water (%)	Methanol (%)	Methylene chloride (%)
1	75%	25	0
2	50	50	0
3	25	75	0
4	20	80	0
5	15	85	0
6	10	90	0
7	5	95	0
8	0	50	50
9	0	0	100
10	0	0	100
11	0	0	100

lution was collected and extracted with hexane. The hexane extract was concentrated and analyzed by GC–MS for breakthrough. The column was then eluted with 2 × 1.5 ml volumes of solvents such as those described in Table II. The resulting fractions were diluted to approximately 475 ml with high-purity water, extracted with hexane, concentrated, and analyzed by GC–MS.

XAD loading and fractionation. A glass chromatography column was packed with 5 ml XAD prepurified resin as a methanol slurry. The resin bed was then washed with 100 ml methanol, followed by 100 ml high-purity water. A 950-ml volume of loading solution was then passed through the column at a rate of 5 ml/min. The post-column solution was collected, extracted into hexane, concentrated, and analyzed by GC–MS for breakthrough. The column was then fractionated with 15-ml volumes of the methanol and methylene chloride solutions listed in Table II. These fractions were then diluted to approximately 475 ml with high-purity water, extracted into hexane, concentrated, and analyzed by GC–MS.

Original elution method. The 1-g C_{18} SPE column was eluted with two successive 1.5-ml aliquots of each of the following methanol–water mixtures: 25, 50, 75, 80, 85, 90, 95 and 100% (v/v) methanol in water. This resulted in eight 3-ml fractions.

Modified elution method. The modified elution scheme eliminates the 100% methanol fraction used in the original method, and adds four fractions, one 50%, and three 100% (v/v) methylene chloride in methanol fractions. The composition of the resulting eleven 3-ml fractions is shown in Table II.

Solvent exchange. Methylene chloride is toxic to aquatic organisms, even at very low concentrations. As a consequence, it must be removed from SPE fractions before the fraction can be tested for toxicity. This should be accomplished without risking the loss of fraction toxicants, and can be achieved by exchanging the methylene chloride fraction into methanol. Exchanging methylene chloride into methanol is relatively easy because of its volatility. The fractions to be exchanged (12 ml) are placed in a centrifuge tube with a PTFE stir bar and an additional 12 ml of methanol. The tube is placed in a 30°C water bath and stirred while a gentle stream of nitrogen is passed over the solution surface. When the volume of the solution reaches 3-ml, the sides are carefully rinsed with 3 additional ml of methanol, and the solution is reduced again to a final 3 ml volume. The recoveries of the test mixture chemicals using this method of solvent exchange are shown in Table III.

TABLE III

RECOVERIES OF TEST MIXTURE CHEMICALS USING SOLVENT EXCHANGE METHOD

Recovery data from one experiment. Abbreviations are listed in Table I.

	Test chemical					
	DEP	NAPH	PHEN	CHRY	HCB	DDE
Recovery (%)	82	69	85	108	81	100

GC–MS analyses. The quantitations of the test mixture chemicals were done using the selected ion monitoring (SIM) mode of the mass spectrometer. For each compound the two major ions and a qualifier ion were acquired. The sediment pore water fractions were analyzed using a full scan mode collecting data on 50–650 amu. Library searches of the resulting spectra were performed automatically by probability matching algorithms and by using the USEPA/NIH/NBS mass spectral library database.

RESULTS AND DISCUSSION

The original phase II TIE procedure of isolating non-polar organic compounds on a C_{18} SPE column was carried out on a sediment pore water. The toxicants were removed by the C_{18} SPE column from the pore water sample, but were not recovered in the methanol–water SPE fractions. However, when methylene chloride was added to the methanol–water elution scheme, toxicity recovered from the column was measured in the eluted fractions. With this initial success in reclaiming difficult to recover toxicity from pore water, further investigation was initiated to determine which elution mixtures of water, methanol and methylene chloride would yield the optimum recovery and separation of compounds over a log K_{ow} range of 2.5 to >5.

Original % recoveries SPE

The % recoveries from C_{18} SPE columns using the original phase II methanol–water elution scheme are listed in Table IV and Fig. 1a. The recoveries of the diethylphthalate, naphthalene and phenanthrene range from acceptable, in the case of phenanthrene, to very good. The percent recovery of the compounds with log K_{ow} greater then five drop off significantly to levels that would probably be undetectable in a toxicity test.

Methods development of elution scheme

The first change that was made to the elution scheme was to increase the number of 100% methanol elutions. When the number was increased to three there was no improvement for the higher log K_{ow} compounds. This was unexpected because these compounds could be eluted from a C_{18} HPLC analytical column using a methanol–water gradient. The next change was to add methylene chloride after the three 100% methanol fractions. The elution profile of this change is shown in Fig. 1b. The higher log K_{ow} compounds did elute in the methylene chloride fractions but there seemed to be two distinct peaks of the same compounds, one in the methanol–water elution portion followed by one in the methylene chloride–methanol fractions. This elution doublet could possibly be the result of a mixed-mode interaction (polar and non-polar) of the C_{18} SPE resin and the test compounds.

Modified % recoveries SPE

Using the modified elution scheme (Table II) the recovery of the higher log K_{ow} compounds increased while the double peak effect observed in the initial

TABLE IV

RECOVERIES OF TEST MIXTURE CHEMICALS USING ORIGINAL AND MODIFIED ELUTION SCHEMES

Recovery data from one experiment. Abbreviations are listed in Table I.

Elution scheme	Sorbent	Test chemical (total % recovery[a])					
		DEP	NAPH	PHEN	CHRY	HCB	DDE
Original	C_{18}	108	116	62	22	52	32
Modified	C_{18}	99	98	109	60	95	76
Modified	XAD-4	112	72	94	101	57	82
Modified	XAD-7	103	95	126	102	109	104
Modified	C_8	104	87	115	77	75	89

[a] Total % recovery = sum of all fraction recoveries.

scheme decreased. The double peak effect for phenanthrene could not be eliminated. The percent recoveries of the sorbent loading mixture compounds are listed in Table IV and Fig. 1c. This elution scheme was repeated using other sorbents commonly used in environmental analyses [17–19]. Two XAD resins, XAD-4 and XAD-7 were evaluated along with C_8 SPE resins. The results of these experiments can be seen in Table IV and Fig. 2. The evaluated resins could be substituted for C_{18} SPE

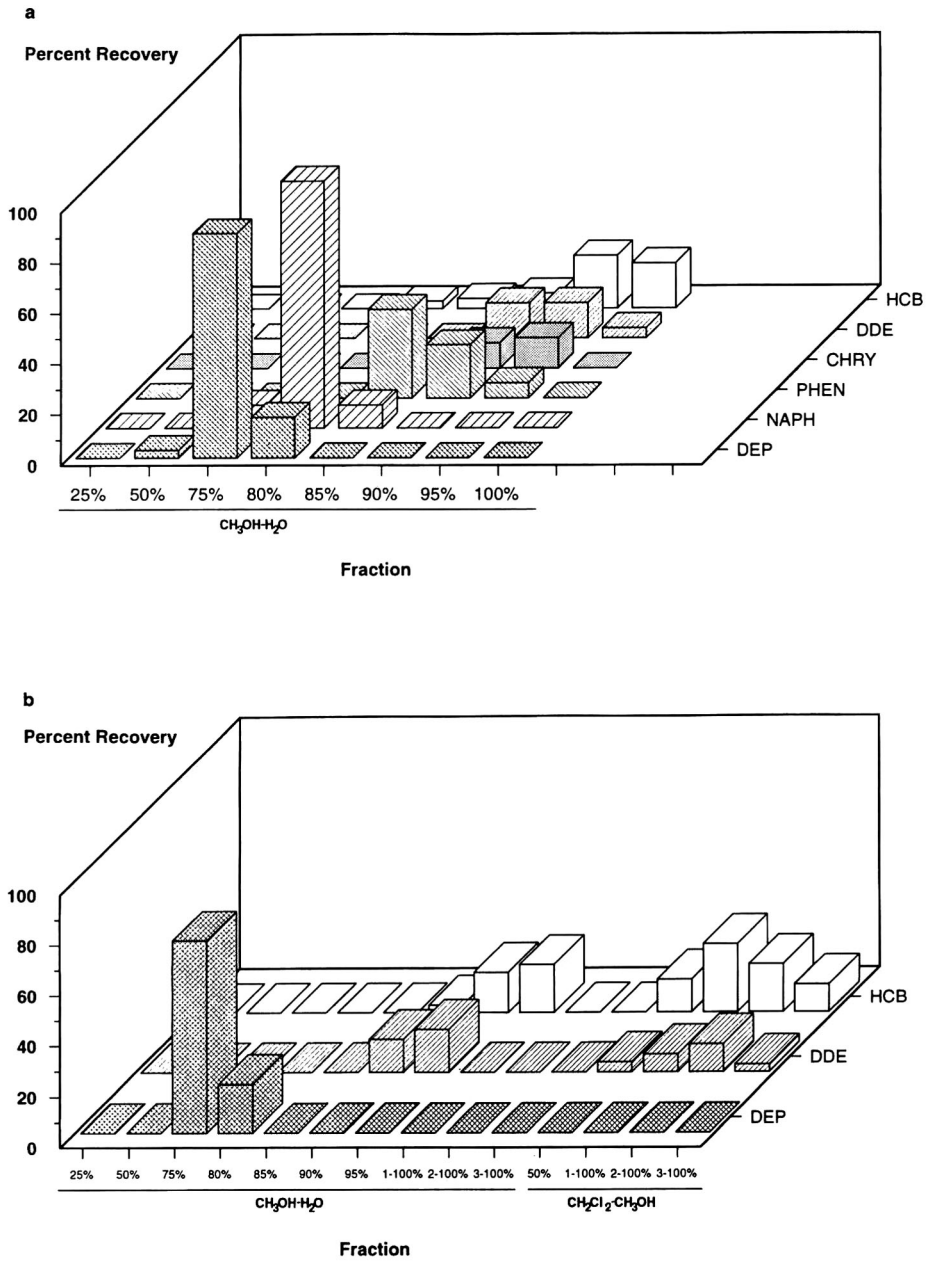


Fig. 1.

(Continued on p. 72)

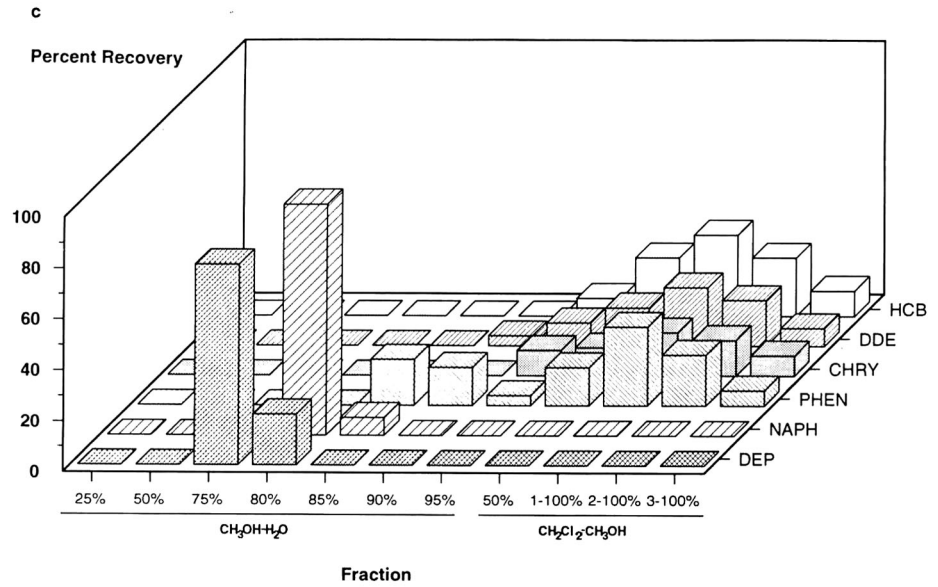


Fig. 1. (a) C_{18} SPE original elution scheme; (b) C_{18} SPE methanol extended elution scheme; (c) C_{18} SPE modified elution scheme.

and reasonable recovery results could be expected. Even though the total recoveries for the test compounds were very good for XAD-7 there was significant compound coelution and each compound eluted in three to five fractions. The ideal resin, and elution scheme would separate each compound from the others, and each chemical would elute in only one fraction.

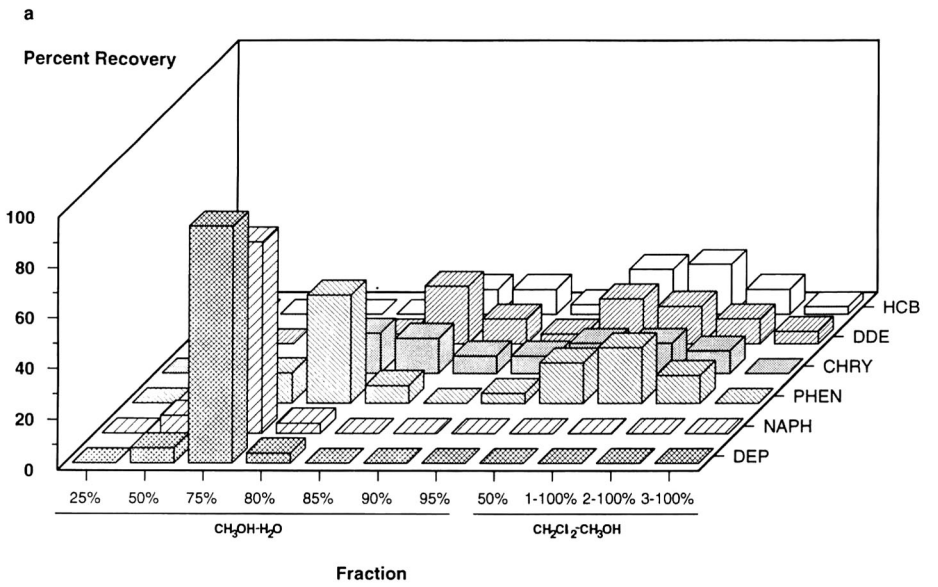


Fig. 2.

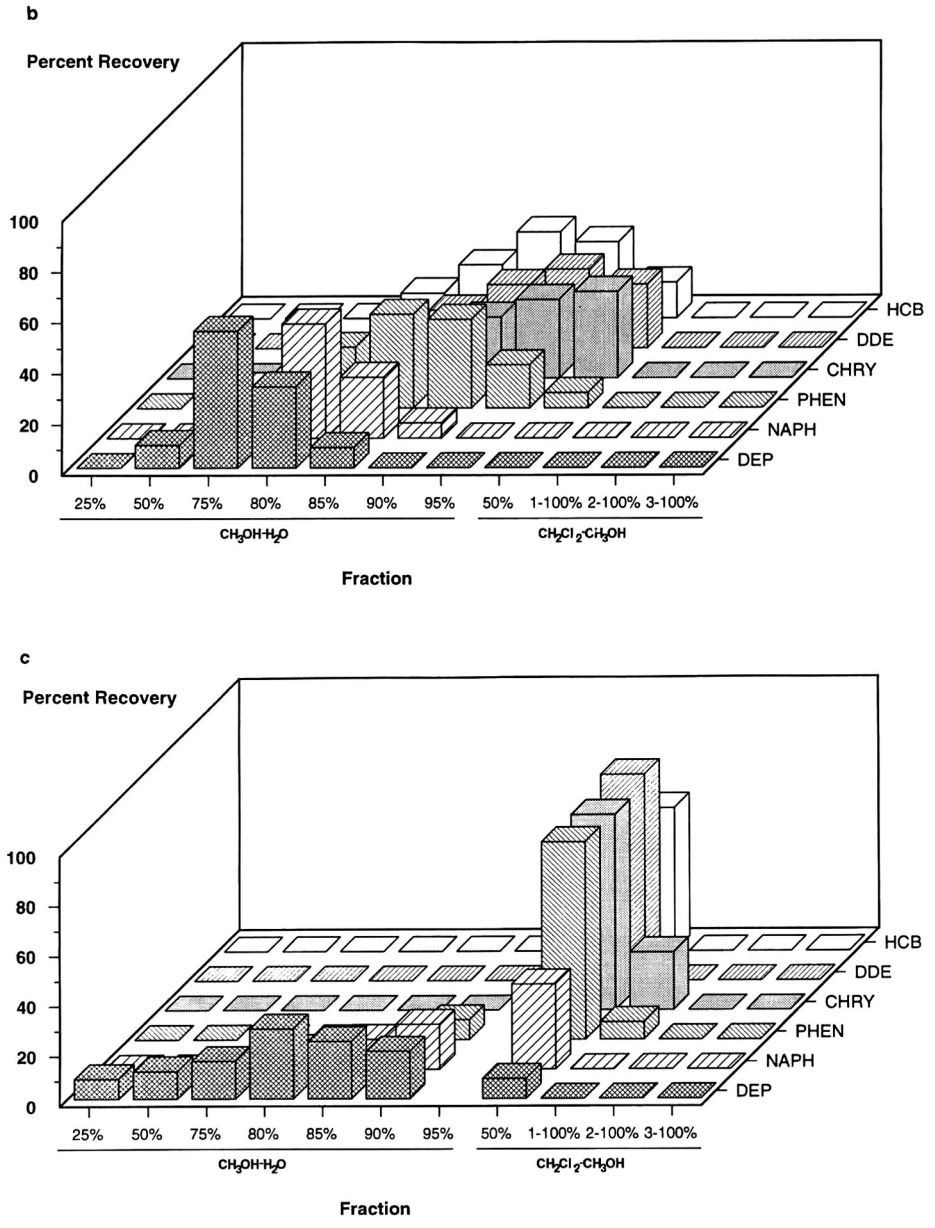


Fig. 2. (a) C_8 SPE modified elution scheme; (b) XAD-7 modified elution scheme; (c) XAD-4 modified elution scheme.

GC–MS identification of fractions of sediment pore water

The fractions collected from the pore water C_{18} SPE fractionation were analyzed using GC–MS. The resulting chromatograms were library searched and a list of tentatively identified compounds was compiled. In general, the results indicate that there are compounds (eleven in this case) that will be eluted from the C_{18} SPE column that cannot be removed by methanol–water elutions. These com-

TABLE V

AVERAGE LOG K_{ow} OF COMPOUNDS IDENTIFIED BY GC–MS IN SEDIMENT PORE WATER C_{18} SPE FRACTIONS

C_{18} SPE fraction	No. of compounds identified	Average log K_{ow}[a]
Methanol–water (25:75)	2	2.2
Methanol–water (50:50)	3	1.3
Methanol–water (75:25)	5	2.1
Methanol–water (80:20)	2	3.4
Methanol–water (85:15)	4	3.7
Methanol–water (90:10)	2	4.2
Methanol–water (95:5)	1	6.1
All methylene chloride fractions	11	7.0

[a] Calculated using the CLOGP program of A. J. Leo, Pomona College, Claremont, CA, Medchem Project, CLOG-3.3 computer program, 1985.

pounds generally have higher log K_{ow} values than the compounds in the methanol–water fractions. A list of all the fractions and the average log K_{ow} of the identified compounds can be seen in Table V.

CONCLUSIONS

The current phase II method for fractionating non-polar organic toxicants in aqueous samples does not effectively fractionate compounds that are highly hydrophobic. Modifications made to the SPE method have been successful in overcoming this limitation. An elution scheme incorporating water, methanol and methylene chloride has been designed that effectively elutes and fractionates compounds over a log K_{ow} range from 2.5 to 6.9. The higher log K_{ow} compounds, however, elute in the same set of fractions. Further fractionation by HPLC is needed to achieve better resolution of these kinds of compounds. Substituting other sorbents for the currently used C_{18} SPE resin has shown that both C_8 SPE and XAD-7 sorbents can be effective for the fractionation of particular kinds of toxicants.

REFERENCES

1 L. P. Burkhard, E. J. Durhan and M. T. Lukasewycz, *Anal. Chem.*, 63 (1991) 277.
2 L. P. Burkhard and G. T. Ankley, *Environ. Sci. Technol.*, 23 (1989) 1438.
3 J. Doi and D. R. Grothe, in G. W. Suter II (Editor), *Aquatic Toxicology and Environmental Fate*. Vol. 11, ASTM STP 1007, ASTM, Philadelphia, PA 1989, p. 123.
4 A. Gasith, K. M. Jop, K. L. Dickson, T. F. Parkerton and S. A. Kaczmarek, in W. J. Adanis, G. A. Chapman and W. G. Landis (Editors), *Aquatic Toxicology and Hazard Assessment*, Vol. 10, ASTM 971, ASTM, Philadelphia, PA, 1988, p. 204.
5 D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures; EPA/600/3-88-034*, US Environmental Protection Agency, Duluth, MN, 1988.
6 D. I. Mount and L. Anderson-Carnahan, *Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Characterization Procedures; EPA/600/3-88-035*, US Environmental Protection Agency, Duluth, MN, 1989.
7 D. I. Mount, *Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Confirmation Procedures; EPA/600/3-88-036*, US Environmental Protection Agency, Duluth, MN 1989.
8 J. R. Amato, D. I. Mount, E. J. Durhan, M. T. Lukasewycz, G. T. Ankley and E. D. Robert, *Environ. Toxicol. Chem.*, 11 (1992) 209.
9 K. M. Jop, T. Z. Kendall, A. M. Askew and R. B. Foster, *Environ. Toxicol. Chem.*, 10 (1991) 981.
10 T. J. Norberg-King, E. J. Durhan and G. T. Ankley, *Environ. Toxicol. Chem.*, 10 (1991) 891.
11 M. K Schubauer-Berigan and G. T. Ankley, *Environ. Toxicol. Chem.*, 10 (1991) 925.
12 G. T. Ankley, A. Katko and J. W. Arthur, *Environ. Toxicol. Chem.*, 9 (1990) 313.
13 M. T. Lukasewycz and E. J. Durhan, *J. Chromatogr.*, 580 (1992) 215.
14 E. Chladek and R. S. Marano, *J. Chromatogr. Sci.*, 22 (1984) 313.
15 M. J. M. Wells, A. J. Rossano, Jr. and E. C. Roberts, *Anal. Chim. Acta*, 236 (1990) 131.
16 R. Frank, R. L. Thomas, H. E. Braun, D. L. Gross and T. T. Davies, *J. Great Lakes Res.*, 7 (1975) 42.
17 P. Van Rossum and R. G. Webb, *J. Chromatogr.*, 150 (1978) 381.
18 S. A. Daignault, D. K. Noot, D. T. Williams and P. M. Huck, *Water Res.*, 22 (1988) 503.
19 G. R. Aiken, E. M. Thurman and R. L. Malcolm, *Anal. Chem.*, 51 (1979) 1799.

Journal of Chromatography, 629 (1993) 75–82
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 670

Fractionation of polar organic extracts of airborne particulate matter using cyanopropyl-bonded silica in solid-phase extraction

L. A. Gundel, K. R. R. Mahanama and J. M. Daisey

Energy and Environment Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720 (USA)

ABSTRACT

A mg-scale fractionation method has been developed for polar organic matter in airborne particles. The method gives reproducibly good recoveries of mass while avoiding the use of water or salts. Cyanopropyl-bonded silica solid-phase extraction (SPE) columns were used to fractionate a mixture of standard compounds and acetone-soluble extracts from particles collected in Elizabeth, NJ, USA and from National Institute of Standards and Technology Standard Reference Material SRM 1649 (urban air particles). Critical factors proved to be reducing the polarity of the extract before its application to the column and pre-wetting the column with *n*-hexane. Ten fractions were eluted with solvent mixtures of increasing polarity, ranging from *n*-hexane to methanol. Blank-corrected mass recoveries were 95 and 98% for the Elizabeth, NJ, USA and SRM 1649 extracts, respectively.

INTRODUCTION

In this paper the term particulate polar matter (PPOM) refers to acetone-soluble material extracted from samples of ambient airborne particles after they have already been extracted with the less polar solvent dichloromethane (or cyclohexane followed by dichloromethane) [1,2]. PPOM accounts for 30 to 60% of the organic-solvent extractable mass of airborne particles [1,2] and 30 to 50% of the direct-acting mutagenic activity in the Ames bioassay with TA-98 [3,4]. However, relatively little work has been done to date to chemically characterize this material or to develop fractionation methods for it. This is because of the difficulties in working with complex mixtures of polar organic compounds.

We chose acetone extracts because some characterization and bioassay data were available for the (unfractionated) acetone extracts used in this study. Methanol extracts have also been characterized to some extent [5,6]. Acetone has been found to extract less inorganic and more organic material from Standard Reference Material SRM 1649, urban air particles, than methanol [5,7]. Acetone extracts of SRM 1649 also had higher mutagenic activity (in revertants per gram of extract) than methanol extracts [5,7].

The goal of the research reported here was to develop a mg-scale fractionation method for particulate polar organics based on polarity, with reproducibly good mass recovery, while avoiding addition of water or salts to the fractions. Evaporation of water or other high-boiling solvents to concentrate samples for bioassay and chemical characterization is very difficult, as is salt removal. Neither normal-phase chromatographic separation on silica or alumina nor reversed-phase separation on C_{18} columns could meet these requirements. Substantial losses of mass could be expected from use of a normal-phase silica or alumina column. Losses of mutagenic activity have also been reported for these sorbents [8,9]. Such losses would not be expected

Correspondence to: L. A. Gundel, Energy and Environment Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720, USA.

for a C_{18} reversed-phase system; however, such systems typically use water as one of the solvents for the separation. Reversed-phase columns also require the presence of buffer salts to control the ionization of polar compounds and thus permit reproducible retention behavior of polar compounds.

Normal-phase separation on cyano-bonded silica was investigated, based on the nature of the organic materials and the need for elution solvents that could be easily evaporated, *e.g.*, *n*-hexane, dichloromethane or methanol [10]. Lafleur and co-workers [9] have reported good recoveries of mass and mutagenic activity for dichloromethane extracts of combustion aerosols which were separated on cyanopropyl solid-phase extraction (SPE) columns. Lafleur and Nakagawa [11] also found good mass recovery when they used cyanopropyl SPE columns to fractionate pyridine extracts of bituminous coal. This is the first study to use cyano-bonded silica for more polar organic materials found in the acetone-soluble fraction of ambient aerosols.

SPE offered the possibility of fractionation of mg quantities of material in a single application. This is in contrast to more time- and solvent-consuming fractionation methods based on fraction collection from cyano-bonded columns in high-performance liquid chromatography [12,13].

EXPERIMENTAL

Sources of particles, sampling, extraction, storage of extracts

Two types of particulate matter were used in this investigation: filter samples of inhalable particulate matter collected in Elizabeth, NJ, USA [14] during the winter of 1983 and National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1649, Washington Urban Air Particulate Material. The Elizabeth samples were collected on pre-cleaned glass fiber filters for 24-h periods over a 6-week period, using a hi-volume air sampler with a size-selective inlet (D_{50} = 15 μm). Individual filters were Soxhlet-extracted sequentially with cyclohexane, dichloromethane, and acetone [1,2,14]. Each extract was filtered and reduced in volume to 10.0 ml using a rotary evaporator. Extract masses were determined with a Cahn microbalance, Model 25, by weighing the residue of duplicate 100-μl aliquots of the extracts taken to dryness on a slide warmer at 45°C. Samples were stored in a freezer at −30°C, in the dark. For this research, a composite sample of the acetone-soluble extracts from Elizabeth, NJ, USA, collected during winter 1983, was prepared based on equal air volumes for each of the 39 days of the sampling period. The total composite mass was 479 mg, corresponding to sampling 44 400 m^3 air over a 6-week period. The SRM 1649 acetone extract was prepared by sequential Soxhlet extraction of a 1-g portion of particles with dichloromethane followed by acetone, filtration and rotary evaporation of excess solvent. Extract samples were stored in the freezer. Previous work had shown that the mass of material extracted with dichloromethane is equivalent to the sum of the masses extracted with cyclohexane followed by dichloromethane [15].

Fractionation on cyanopropyl-bonded phase columns

SPE solid-phase extraction columns containing 500 mg of cyanopropyl-bonded silica packing (J.T. Baker) were used. They were pre-cleaned with 10 ml of chromatographic-grade solvents, in order: *n*-hexane, ethyl acetate, dichloromethane, and methanol. The columns were covered loosely with aluminum foil and dried overnight. Prior to sample application, the columns were wetted with 2 ml of *n*-hexane. The acetone extract of the Elizabeth sample was evaporated to near dryness, redissolved in methanol and then evaporated to near dryness again. This was done to remove acetone which has appreciable ultraviolet absorbance and interferes with characterization by high-performance liquid chromatography. Methanol, dichloromethane and *n*-hexane were added to the dried extract, in that order, so that the final solvent proportions were 1:1.2:1.4. This mixture of solvents dissolved the extract completely and kept the extract in a narrow band at the top of the column.

Before application to the column the mass density of the mixture was determined for duplicate dried 100-μl aliquots, using a Cahn microbalance. An amount of 4 mg of extract in 200 μl of solvent mixture was applied to the top of a pair of coupled columns which had been wetted with *n*-hexane. The loaded columns dried in air overnight. A series of elution solvent mixtures of increasing polarity was then passed through the coupled columns, and the eluent fractions were collected and evaporated to

dryness. Methanol (500 μl) was added to each fraction and the dry mass of two 100-μl portions was determined. The elution solvents were: *n*-hexane, 25% dichloromethane in *n*-hexane (2 aliquots), 100% dichloromethane (2 aliquots), 2%, 5%, 15% and 40% methanol in dichloromethane, and 100% methanol. The aliquots were each 2 ml, except for the methanol which was 3 or 6 ml. Air pressure was applied to the top of the coupled columns to keep the solvent flow-rate at about 2 ml/min.

The fractionation and mass determination were done with three separate aliquots of the Elizabeth winter 1983 extract. The fractions were labelled A–J in order of increasing elution solvent polarity. Column blanks were eluted the same way and the mass in each recovered fraction determined for a blank correction. The fractions were characterized by HPLC with ultraviolet (UV) detection. A 4-mg sample of the acetone extract of NBS 1649 particles was also fractionated and characterized as described above. The capacity of 500-mg SPE columns was determined by adding up to 6.2 mg of the Elizabeth extract mixture and assessing the quality of the separation visually.

The fractionation procedure was performed on a mixture of these standard compounds (obtained from Aldrich): 5-nitrovanillin, vanillin, 2-naphthoic acid, 1,4-dihydroxynaphthalene, 5-nitroquinoline, 2-nitro-6H-dibenzo[*b*,*d*]pyran-6-one (6-nitro-3,4-benzocoumarin), 2-nitronaphthalene, 5,6-benzoquinoline, acridine, pyrene carboxaldehyde and dioctylphthalate. The standards were prepared in either methanol or acetonitrile, but the mixture was evaporated to dryness and reconstiuted in 0.45 ml of *n*-hexane, dichloromethane and methanol in the proportions given above, before application to a single clean, *n*-hexane-wetted cyano SPE column. Elution and analysis procedures were the same as for the extracts of ambient particles. Two aliquots of the standard mixture were fractionated. In one experiment, evaporation losses were evaluated by determining recovery of the standard compounds after reconstitution and before fractionation. In another experiment the Elizabeth acetone extract was spiked with the standard mixture and fractionated.

High-performance liquid chromatography

Standard compounds, whole extracts and fractions were analyzed on a Hewlett-Packard Model 1090M high-pressure liquid chromatograph equipped with a DR 5 solvent delivery system, a diode array UV–Vis detector, a fluorescence detector (Model 1046A) and Model 79994A LC Workstation software. Samples were chromatographed on a Vydac 201TP52 C_{18} reversed-phase microbore column, 15 cm × 2.1 mm I.D. and 5 μm diameter particles, using gradient elution. The analytical column was preceded by a guard cartridge column filled with 10 μm diameter Vydac 201TP packing material (purchased from Alltech). A 5-μl injection loop was used. The initial solvent composition was 5% acetonitrile in water. From 3 to 13 min the solvent composition was changed linearly to 100% acetonitrile and held there for 5 min. Gradient reversal and column equilibration were complete 25 min after injection. Flow through the column was 0.3 ml/min. Three absorbance wavelengths were used for detection: 205, 230 and 254 nm. The fluorescence detector used an excitation wavelength of 230 nm, and all emitted light above 305 nm was collected.

Retention times were determined for the polar aromatic standard compounds at about 0.4 mg ml^{-1} each in a methanol–acetonitrile mixture (1:5, v/v): 5-nitrovanillin, 1.6 min; vanillin, 4.2 min; 2-naphthoic acid, 6.5 min; 1,4-dihydroxynaphthalene, 7.6 min; 5-nitroquinoline, 8.4 min; 2-nitro-6H-dibenzo[*b*,*d*]pyran-6-one, 12.4 min; 2-nitronaphthalene, 12.8 min; 5,6-benzoquinoline, 13.6 min; acridine, 14.2 min; pyrene carboxaldehyde, 14.6 min; and dioctylphthalate, 16.8 min. Retention times for 5-nitrovanillin, vanillin, naphthoic acid and dihydoxynaphthalene depended on the condition of both the analytical and guard columns and use of freshly filtered and degassed elution solvents. The gradient elution program was modified as necessary to ensure reproducible retention times of standard compounds before the extracts of ambient particles or their fractions were analyzed.

RESULTS

In developing this fractionation method, the behavior of the colored Elizabeth extract on the cyano SPE columns was monitored using various proportions of hexane, dichloromethane and methanol as elution solvents. Initially, visual monitoring of the movement of colored bands on the column and the

mass distribution in the resulting fractions were used to assess the success of the fractionation. Finally, characterization by HPLC was used to assess polarity differences among UV-absorbing components of the fractions. The choice of elution solvents was based on low boiling points, relative chemical inertness and UV absorbance. All fractions were redissolved in methanol because of its UV transparency and compatibility with reversed-phase HPLC. Development of the fractionation method started with actual acetone extracts of ambient particles rather than with standard compounds because very little data is available about the chemical composition of such polar extracts of ambient air particles.

At first we intended to separate the extract mass into four fractions, if possible. We found that ten fractions of increasing polarity led to three separate mass recovery maxima, centered on fractions B, D and H (Table I). Table I shows that B was colorless, but both D and H were yellow. Each group of fractions was separated by at least one fraction which had much lower mass. Such a mass distribution showed promise for future bioassay and chemical characterization studies.

Once the desired fractionation mass distribution had been achieved, three pairs of coupled (500 mg) cyano-bonded silica packed columns were used to fractionate three aliquots (4 mg each) of the acetone extract of the Elizabeth winter particle samples. Critical factors in successful fractionation proved to be reduction of the polarity of the extract before its application to the column and wetting the column with *n*-hexane before application of the extract. The blank-corrected mass distribution is displayed in Table I for the three aliquots of Elizabeth Winter 1983 particles. The total mass recovery, with blank correction, averaged 96 ± 5%. The most polar fractions G, H, I and J, accounted for 61% of the recovered mass. The observed differences in mass distribution among the replicates reflect differences in elution flow-rate, one done at 2 ml min^{-1} and two at about 4 ml min^{-1}. For the latter two, 8 and 6 ml methanol were used, respectively, for the final fraction J, whereas the first used only 3 ml methanol. Larger methanol volumes yielded only a few percent better recoveries.

Each fraction from the first of the three fractionations was characterized by HPLC with diode array detection at 205, 230 and 254 nm. The chromatograms for 205 nm are shown in Fig. 1. Absorbance intensity decreased in the order 205 > 230 > 254 nm for all peaks. The unfractionated extract had two peaks at widely separated retention times, indicating the presence of two strongly-absorbing polarity groups within the acetone extract. The second fraction, B, showed a strong absorbance at the same retention time and wavelength as observed for the polar fraction in the preliminary fractionation by solvent polarity [7]. The fourth fraction, D, had no appreciable absorbance although it contained 10%

TABLE I

FRACTIONATION OF THE ACETONE EXTRACT OF AMBIENT PARTICLES FROM ELIZABETH, NJ, USA

Fraction	Solvent	Color	Intensity[a]	Mass recovery, %[b]	
				Average	S.D.
A	Hexane	Clear	0	1.2	0.1
B	25% $MeCl_2$	Clear	0	8.6	0.4
C	25% $MeCl_2$	Faint	0.5	2.7	1.7
D	$MeCl_2$	Light yellow	3	9.8	1.5
E	$MeCl_2$	Light yellow	2	5.8	0.4
F	2% MeOH	Pale	1	4.7	1.2
G	5% MeOH	Yellow	3	16.2	4.0
H	15% MeOH	Yellow	5	31.4	7.1
I	40% MeOH	Light yellow	1.5	13.1	1.2
J	MeOH	Clear	0	6.7	2.6
Total recovery				96	5

[a] Visual estimate; a value of 5 was arbitrarily assigned to the fraction with the most intense color.

[b] Blank-corrected recovery, %.

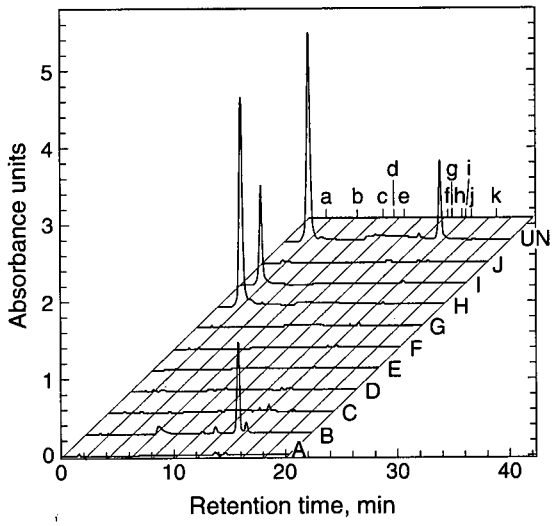


Fig. 1. Reversed-phase chromatograms of the acetone extract of Elizabeth ambient particles. The ordinate shows absorbance at 205 nm in absorbance units. The top chromatogram indicates the retention times for the standard compounds: a = 5-nitrovanillin; b = vanillin; c = 2-naphthoic acid; d = 1,4-dihydroxynaphthalene; e = 5-nitroquinoline; f = 2-nitro-6H-dibenzo[*b*,*d*]pyran-6-one; g = 2-nitronaphthalene; h = 5,6-benzoquinoline; i = acridine; j = 1-pyrenecarboxaldehyde, and k = dioctylphthalate. The unfractionated extract is labelled as UN; fractions are identified by capital letters at the right of each chromatogram.

of the mass. Fractions H and I had absorbance peaks similar to that seen for the very polar fraction VP [7]. Other fractions had no appreciable absorbance. Based on comparison of retention times to those of standards, the first fractions were much less polar than the later eluting fractions.

The acetone extract of SRM 1649 had a mass distribution similar to that for Elizabeth, as indicated in Table II. Blank-corrected mass recovery was 98%. Absorbance chromatograms at 205 nm are shown in Fig. 2. The total extract mass separated into three broad groups, centered on fractions B, D and H. The mass distribution was similar to that observed for Elizabeth, but the chromatograms did not show much absorbance for the least polar mass peak (B).

The capacity limit of single 500 mg cyanopropyl-bonded silica columns was determined to be between 5.7 and 6.2 mg for the Elizabeth extract. The visually-determined integrity of the fractionation procedure was beginning to deteriorate at 6.2 mg, and no heavier loadings were attempted.

After the acetone extracts of ambient particulate matter had been fractionated and analyzed, the procedure was tested with a standard mixture containing polar aromatic compounds. Compounds were selected on the basis of their polarity and the possiblity of their presence in ambient particulate matter. They also had to have appreciable UV absorbance or fluoresence. Included in the mixture was a compound recently identified as a potent mutagen in dichloromethane extracts of ambient air particles, 2-nitro-6H-dibenzo[*b*,*d*]pyran-6-one [16]. All compounds except 5-nitrovanillin were eluted in fractions A and B. Less than 1% of the applied

TABLE II

FRACTIONATION OF THE ACETONE EXTRACT OF AMBIENT PARTICLES-SRM 1649

Fraction	Solvent	Color	Intensity[a]	Mass recovery, %[b]
A	Hexane	Clear	0	6.8
B	25% $MeCl_2$	Pale	1	8.9
C	25% $MeCl_2$	Clear	0	2.9
D	$MeCl_2$	Faint	0.5	10.3
E	$MeCl_2$	Clear	0	6.7
F	2% MeOH	Clear	0	2.8
G	5% MeOH	Light yellow	2	13.1
H	15% MeOH	Yellow	4	26.2
I	40% MeOH	Pale	1	13.3
J	MeOH	Clear	0	8.8
Total recovery				98

[a] Visual estimate; a value of 4 was arbitrarily assigned to the fraction with the most intense color.
[b] Blank-corrected recovery, %.

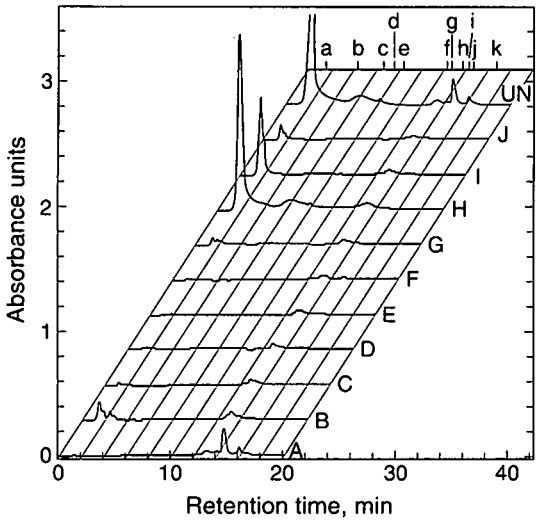


Fig. 2. Reversed-phase chromatograms of the acetone extract of SRM 1649 ambient particles. The ordinate shows absorbance at 205 nm in milliabsorbance units. The top chromatogram indicates the retention times for the standard compounds: a = 5-nitrovanillin; b = vanillin; c = 2-naphthoic acid; d = 1,4-dihydroxynaphthalene; e = 5-nitroquinoline; f = 2-nitro-6H-dibenzo[*b*,*d*]pyran-6-one; g = 2-nitronaphthalene; h = 5,6-benzoquinoline; i = acridine; j = 1-pyrenecarboxaldehyde, and k = dioctylphthalate. The unfractionated extract is labelled as UN; fractions are identified by capital letters at the right of each chromatogram.

mass of any compound was found in any other fraction, with typically at least two thirds of the mass of each compound found in fraction A. 5-Nitrovanillin was not recovered from any of the ten fractions in either attempt. The recovery data from the evaporation and fractionation procedures are given in Table III. Compounds are listed in order of increasing retention time (decreasing polarity) on the reversed-phase HPLC column.

Six compounds showed substantial losses in the evaporation step: three of the four nitro compounds, plus vanillin, 2-naphthoic acid and 1,4-dihydroxynaphthalene. Four are naphthalene derivatives which are fairly volatile; the other two are benzene derivatives with multiple polar groups. These losses were reflected in lower than average fractionation recoveries. The losses were more severe in the first fractionation. The two fractionation experiments differed in whether or not the column was air-dried prior to elution and the amounts of applied material. Low applied masses and a degenerating guard column in the HPLC led to no detection of the first four compounds in the first fractionation. The second fractionation used about four times the mass used in the first fractionation, and somewhat higher recoveries were obtained overall.

TABLE III

EVAPORATION AND FRACTIONATION DATA FOR STANDARD COMPOUNDS

Compound	Intitial mass (μg)			Recovery (%)		
	Evap.[a]	Frac. 1[b]	Frac. 2[c]	Evap.[a]	Frac. 1[b]	Frac. 2[c]
5-Nitrovanillin	4.6	4.6	18.5	71	–	–
Vanillin	4.2	4.2	17.5	39	–	81
2-Naphthoic acid	0.87	0.87	3.5	57	–	82
1,4-Dihydroxynaphthalene	1.6	1.6	6.5	27	–	59
5-Nitroquinoline	4.6	4.6	4.6	60	66	80
2-Nitro-6H-dibenzo[*b*,*d*]pyran-6-one	1.4	1.4	5.4	115	86	91
2-Nitronaphthalene	1.8	1.8	7.1	52	106[d]	78
5,6-Benzoquinoline	1.8	1.8	7.2	112	67	90
Acridine	1.9	1.9	7.7	89	72	90
1-Pyrenecarboxaldehyde	1.6	1.6	6.4	97	52[d]	90
Dioctylphthalate	3.8	3.8	15.2	117	105	96.3
Average ± S.D.				76 ± 32	79 ± 21	84 ± 10

[a] Standard mixture was evaporated to dryness in a water bath and reconstituted in methanol.

[b] Standard mixture was reconstituted in *n*-hexane, dichloromethane and methanol, and fractionated. Column was air dried before elution, as was done for extracts of ambient particles.

[c] Standard mixture was reconstituted in *n*-hexane, dichloromethane and methanol and fractionated without column drying before elution.

[d] These values are less certain because of the condition of the HPLC column.

The averaged recovery results for the other compounds are statistically indistinguishable for the two experiments. The effect of air-drying does not appear to be significant. Losses of the standard compunds appear to be due largely to evaporative losses rather than irreversible adsorption or reaction on the cyanopropyl-bonded silica of the SPE column.

A mixture of the Elizabeth acetone extract and the standard compounds was also fractionated and analyzed by HPLC. The resulting absorbance chromatograms resembled the sum of the independent chromatograms; *i.e.*, there was no apparent interaction between the two sample types. Retention times and recoveries were not substantially changed.

DISCUSSION AND CONCLUSIONS

Cyanopropyl-bonded silica SPE columns proved capable of fractionating mg quantities of acetone extracts of airborne particulate matter with good recovery of mass without addition of water or buffer salts. When eluted using normal-phase chromatography, with solvent mixtures of increasing polarity, the resultant fractions increased in polarity, as judged by HPLC analysis. This is consistent with the results of fractionating pyridine extracts of coal using the same type of SPE columns [11].

The observation that the acetone extracts of ambient particles redissolved in methanol can be explained by noting that methanol is more polar than acetone. Fractionation of the acetone extract has been possible because of the selective elution of components of the material from the cyano SPE adsorbent in a series of solvent mixtures, most of which are much less polar than the extraction solvent, acetone. Since the parent pollution particles had already been extracted with dichloromethane, this result may appear surprising. However, the original two-step extraction process had dissolved species which may have been strongly adsorbed onto the particulate matrix. Once desorbed from that matrix they may have been more soluble in solvent mixtures of lower polarity than they were when sorbed onto the matrix. We found that the ten-fraction separation was only possible when *n*-hexane, a non-polar solvent, was used, along with dichloromethane, to decrease the polarity of the ambient samples and allow adsorption of the polar components onto the cyanopropyl matrix before fractionation could begin. A stronger adsorbent such as non-bonded silica would probably have irreversibly adsorbed the species of interest, perhaps parallelling the earlier adsorption of the polar species to their parent particulate matrix.

The standard compounds tested in this study were apparently less polar or more soluble in *n*-hexane and dichloromethane than the acetone extracts of ambient particulate matter for which the technique has been developed. All the standards eluted before the bulk of the mass of either extract of airborne particles. An earlier study using preparative HPLC with a cyano column [12] found vanillin in the fourth of seven fractions of increasing polarity. (In that study the most polar fractions were not extensively characterized due to the limited recovery of very polar material from gas chromatographic columns.) Vanillin was one of the two most polar compounds tested here, as judged from reversed-phase HPLC retention times. Non-polar and moderately polar species such as vanillin and the nitrated low-molecular-mass polycyclic aromatic hydrocarbons would have been removed from the ambient particles during the first extraction step in dichloromethane.

The technique reported here is easy, fast and inexpensive when compared to fractionation by preparative-scale HPLC. Mass distributions for both acetone extracts of ambient particles and a mixture of polar standard compounds were quite reproducible. The cyano SPE columns showed no evidence for destruction or irreversible adsorption of components in the polar extracts of urban airborne particulate matter.

ACKNOWLEDGEMENTS

The authors thank Michael Henry and William Leister of J.T. Baker, Inc., and Roger Atkinson of the Statewide Air Pollution Research Center, University of California, Riverside, CA, USA, for useful information and suggestions. This work was supported by Grant R-815755-02-0 from the U.S. EPA, the Director, Office of Energy Research, Office of Health and Environmental Research, Human Health and Assessments Division, the U.S. Department of Energy, under Contract No. DE-AC03-76SF00098, and the Ford Motor Company.

The information in this paper has not been reviewed by the U.S. EPA and does not necessarily reflect the views of that organization.

REFERENCES

1 J. M. Daisey, R. J. Hershman and T. J. Kneip, *Atmos. Environ.*, 16 (1982) 2161–2168.
2 J. M. Daisey, in P. J. Lioy and J. M. Daisey (Editors), *Toxic Air Pollution, A Comprehensive Study of Non-criteria Air Pollutants*, Lewis, Chelsea, 1987, pp 47–65.
3 J. M. Daisey, T. J. Kneip, I. Hawryluk and F. Mukai, *Environ. Sci. Technol.*, 14 (1980) 1487–1490.
4 T. B. Atherholt, T. B. McGarrity, J. B. Louis, L. J. McGeorge, P. J. Lioy, J. M. Daisey, A. Greenberg and F. Darack, in M. D. Waters, S. S. Sandhu, J. Lewtas, L. Claxton, G. Strauss and S. Nesnow (Editors), *Short-Term Bioassays in the Analysis of Complex Environmental Mixtures IV*, Plenum, New York, 1985, pp. 211–231.
5 M. Zinbo, D. Schuetzle, D. H. Hsieh, N. Y. Kado, J. M. Daisey and L. A. Gundel, *Anal. Sci.*, 8 (1992) 461–468.
6 Y. Yokouchi and Y. Ambe, *Atmos. Environ.*, 20 (1992) 1727–1734.
7 L. A. Gundel, J. M. Daisey, L. R. F. Carvalho, N. Y. Kado and D. Schuetzle, *Environ. Sci. Technol.*, (1992) submitted.
8 A. Sicherer-Roetman, M. Ramlal, C. E. Voogd and H. J. Th. Bloemen, *Atmos. Environ.*, 22 (1988) 2803–2808.
9 A. L. Lafleur, A. G. Braun, P. A. Monchamp and E. F. Plummer, *Anal. Chem.*, 58 (1986) 568–572.
10 M. Zeif and R. Kiser, *Am. Lab.*, January (1990) 70-83.
11 A. L. Lafleur and Y. Nakagawa, *Fuel*, 68 (1989) 741–752.
12 T. J. Mast, D. P. H. Hsieh and J. N. Seiber, *Environ. Sci. Technol.*, 18 (1984) 338–348.
13 J. C. Crowley, S. Siggia and P. C. Uden, *Anal. Chem.* 52 (1980) 1224–1228.
14 P. J. Lioy and J. M. Daisey (Editors), *Toxic Air Pollution, A Comprehensive Study of Non-criteria Air Pollutants*, Lewis, Chelsea, 1987.
15 J. M. Daisey, J. D. Spengler and P. Kaarakka, *Environ. Intern.*, 15 (1989) 435–442.
16 D. Helmig, J. Arey, W. P. Harger, R. Atkinson and J. Lopez-Cancio, *Environ. Sci. Technol.*, 26 (1992) 622–624.

Journal of Chromatography, 629 (1993) 83–87
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 554

Application of solid-phase extraction in the determination of U-82217 in rat serum, urine and brain

W. Z. Zhong

Drug Metabolism Research, Upjohn Laboratories, Kalamazoo, MI 49001 (USA)

ABSTRACT

The techniques of solid-phase extraction (SPE) were applied in the analytical method development for the determination of U-82217, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-[(4-methoxyphenyl)methyl]-imidazo[1,5-*a*]quinoxalin-4(5*H*)-one, in rat serum, urine and brain. Samples of serum, urine or brain homogenate containing U-82217 were loaded on C_{18} SPE columns and eluted with acetonitrile (300 μl). The prepared samples were analyzed by reversed-phase HPLC using an ODS column with a mobile phase of acetonitrile–water (45:55, v/v) containing 0.12% of acetic acid (pH 6.0 ± 0.1). The UV absorbance of the column effluent was monitored at a wavelength of 318 nm. The absolute extraction recovery from serum, urine and brain samples was *ca.* 90%. Linear calibration graphs were obtained over the ranges 5 ng/ml–20 μg/ml (serum), 20 ng/ml–20 μg/ml (urine) and 50 ng/g–200 μg/g (brain). The intra- and inter-assay precision and accuracy were all found to be < 13% at the concentrations evaluated. The strategy in SPE development and the application of this method to the determination of U-82217 in rat serum and brain for a pharmacokinetic study are also discussed.

INTRODUCTION

Solid-phase extraction (SPE) methods have been extensively used in the extraction of chemicals from biological samples. Highly purified and concentrated isolates for chromatographic analysis can be achieved by the selective extraction with desired sorbents to yield chromatograms with minimal interferences and improved sensitivity [1]. Therefore, the SPE method is useful in bioanalytical method development. The method for the determination of U-82217 in rat serum, urine and brain is a typical example of the application of SPE in biomedical analysis. U-82217, 3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-[(4-methoxyphenyl)methyl]-imidazo-[1,5-*a*]quinoxalin-4(5*H*)-one, is under preclinical evaluation as a potential hypnotic compound. U-82217 is an oxadiazole-substituted imidazobenzodiazepine compound which has demonstrated reduced physical dependence-inducing properties compared to benzodiazepine agonists, minimal amnesia-inducing effects and high potency on locomotor-based hypnotic tests in animal models [2]. A number of analytical methods have been reported for the determination of other imidazobenzodiazepines in biological samples [3–7]. Most of them were for plasma samples and used a liquid–liquid extraction procedure for isolating the compounds of interest from the biological matrix. An HPLC method with UV detection using an SPE procedure for the determination of U-82217 in rat serum, urine and brain was therefore developed to support the evaluation of oral bioavailability and pharmacokinetic characteristics and initial toxicokinetic properties of U-82217 in animal models. In this paper the analytical method development and validation for the determination of U-82217 in biological matrices, particularly the development of SPE procedures, are discussed.

Correspondence to: W. Z. Zhong, Drug Metabolism Research, Upjohn Laboratories, Kalamazoo, MI 49001, USA.

EXPERIMENTAL

Reagents and materials

U-82217 and the internal standard (I.S.),

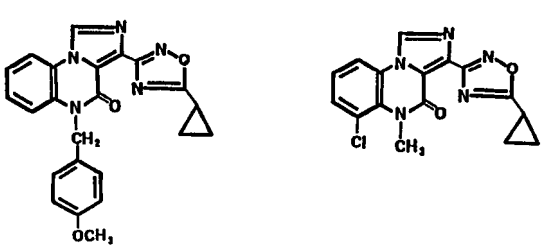
U-80447 (Fig. 1) were provided by Upjohn (Kalamazoo, MI, USA). HPLC-grade acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA) and analytical-reagent grade acetic acid, ammonia solution and potassium phosphate (dibasic) from Mallinckrodt (Paris, KY, USA). Purified water was produced with a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

Chromatography

The liquid chromatographic system consisted of a Waters (Milford, MA, USA) M-6000A pump, a Kratos (Ramsey, NJ, USA) Spectroflow 783 variable-wavelength UV detector set at 318 nm, a Perkin-Elmer (Norwalk, CT, USA) ISS-100 Autosampler fitted with a 200-μl sample loop, and an ODS column (250 × 4.6 mm I.D., 5 μm particle size) (Jones Chromatography, Littleton, CO, USA) protected by a Pelliguard ODS guard column (50 × 2.1 mm I.D., 32 μm particle size) (Whatman, Clifton, NJ, USA). The mobile phase was a mixture of 450 ml of acetonitrile, 550 ml of water and 1.2 ml of acetic acid with an apparent pH of 6.0 ± 0.1 adjusted with ammonia solution, and was then filtered and degassed with helium prior to use. The chromatographic system was operated at ambient temperature (21–23°C) with an eluent flow-rate of 1.0 ml/min. Quantification was accomplished based on the peak-height ratio of drug to I.S. using a Harris computer system.

Serum extraction

Twelve C_{18} SPE columns (100 mg/1.0 ml) (Varian, Harbor City, CA, USA) placed on the vacuum extraction manifold (Supelco, Bellefonte, PA, USA) were prewashed with one column volume of acetonitrile followed by one column volume of 0.1 *M* K_2HPO_4 solution. Unknown serum samples (1ml), each mixed with 50 μl of 10 μg/ml I.S. solution, were loaded onto SPE columns with a vacuum of 86 kPa. After vacuum aspiration for 5 min at *ca.* 27 kPa, the SPE columns were rinsed with 100 μl of acetonitrile–water (30:70,v/v) followed by 2 ml of 0.1 *M* K_2HPO_4 solution (86 kPa). The columns were dried with vacuum aspiration (*ca.* 27 kPa) for 10 min. U-82217 and the I.S. were then eluted from the column with 300 μl of acetonitrile by applying a slow uniform pressure to the top of the column using nitrogen (about 0.2 kg/cm^2). Each eluate was collected in a 2-ml autosampler vial and mixed with 200 μl of purified water. A 50-μl volume of the mixture was injected into the HPLC system for analysis.



U-82217 **I.S.**

Fig. 1. Structures of U-82217 and the I.S. (U-80447).

Urine extraction

A 1-ml volume of unknown urine sample mixed with 100 μl of 10 μg/ml I.S. solution was transferred to the prewashed SPE column as described above. The SPE column was rinsed with 150 μl of acetonitrile–water (30:70, v/v) under gravity flow (without vacuum). The remainder of the SPE procedure was the same as described for serum except that 700 μl instead of 200 μl of water were mixed with the 300 μl of acetonitrile extract for final HPLC analysis.

Brain extraction

Unknown brain samples were prepared by homogenizing accurately weighed rat brain sample (*ca.* 200 mg) in a 5-ml grinding chamber with 1 ml of acetonitrile–water (50:50, v/v). The homogenate was combined with 1 ml of water used for rinsing the grinder piston and vortex mixed for 30 s. A 1-ml volume of the brain homogenate was transferred into a 1.5-ml micro centrifuge tube, mixed with 50 μl of I.S. working solution (10 μg/ml) and centrifuged at 750 *g* for 1 min in a Brinkmann (Westbury, NY, USA) Model 5415 micro centrifuge. The supernatant of brain homogenate was then transferred into a C_{18} SPE column. The remainder of the SPE procedure was the same as that for the serum extraction.

Validation

The assay validation was similar to the analytical

method validation procedures described by Shah *et al.* [8] for bioavailability, bioequivalence and pharmacokinetics studies. Briefly, to determine the linear range of this method, freshly prepared fortified standards of U-82217 were analyzed on four different days. The limit of quantification (LOQ) was estimated by analyzing fortified serum samples at the presumed LOQ in five replicates to determine if it had acceptable precision and accuracy (<20%). The precision and accuracy of the method were evaluated at three concentrations (low, medium and high). The intra-assay precision was determined by analyzing five fortified serum samples at each concentration on the same day, and the inter-assay precision was obtained by analyzing one fortified serum sample at each concentration on four different days. The absolute extraction recovery was determined at three concentrations for U-82217 and the I.S. Samples at each concentration were extracted as outlined for unknown samples and analyzed in four replicates. The peak heights of the extracted samples were compared with those of unextracted external reference standards containing the corresponding concentrations. In all instances, the means, standard deviations (S.D.) and relative standard deviations (R.S.D.) were calculated. A p-value of <0.05 was considered significant in statistical analysis.

RESULTS AND DISCUSSION

Solid-phase extraction development

Extraction is still the one of the most important preparation procedures for the assay of drugs in biological samples. An efficient extraction method can improve the assay precision and accuracy and also the sensitivity. It is well known that proteins are major components that must be removed from biological samples before chromatographic analysis. The solid-phase extraction technique has been proved to be one of the best approaches for removing large amounts of protein from biological samples. Owing to their high molecular masses, most proteins are exposed to a minimum of active functional groups on the sorbent surface and pass unretained, particularly through non-polar SPE columns. The retained proteins can be easily eluted by rinsing the SPE column with water or buffer solutions. Therefore for this application, C_8, C_{18} and phenyl SPE columns were evaluated to determine their extraction efficiencies. Among these, the C_{18} column proved to be the most satisfactory phase as far as the extraction recovery of U-82217 was concerned. Further, a C_{18} column provided relatively cleaner background of chromatographic profiles with minimum impurity front from endogenous components of biological samples compared with phenyl and C_8 columns. In general, the smaller volume of solvent used for the elution of the compounds of interest from the SPE column produced cleaner extracts if an adequate recovery of U-82217 and the I.S. can be achieved. The 100 mg of sorbent were sufficient to retain the compounds and provided efficient elution with a minimum volume of solvent (300 μl) so that the compounds could be concentrated for HPLC without the need for evaporation. Approximately 80% extraction recovery was obtained when using 300 μl of acetonitrile to elute U-82217 and the I.S. The recovery was further improved to 90% when the SPE column was conditioned with 2 ml of 0.1 M K_2HPO_4 prior to the elution step, which provided a basic environment to facilitate complete elution of the compounds of interest. A large impurity front from endogenous urine components was observed following the extraction procedure developed for serum samples. When the SPE column was rinsed with 150 μl of acetonitrile–water (30:70, v/v) under gravity flow, most of the endogenous urine components were eliminated without reducing the extraction recovery. The solvent used for extracting U-82217 from brain was acetonitrile–water (50:50 v/v), 1 ml of which was sufficient to obtain an extraction recovery greater than 90%. The extract was then purified through the SPE column, yielding a clean chromatogram with no interfering peaks at the retention volumes of U-82217 and the I.S.

The average absolute extraction recoveries were evaluated for both U-82217 and the I.S. at concentrations of 0.01, 0.5 and 10 μg/ml for serum, 0.02, 0.5 and 10 μg/ml for urine and 0.1, 5 and 100 μg/g for brain. The overall average ($n=4$) extraction recoveries for U-82217 ranged from 93.5 ± 3.0 to $95.6\pm2.6\%$, 91.2 ± 3.3 to $94.0\pm3.5\%$ and 88.7 ± 6.1 to $95.4\pm4.8\%$, for serum, urine and brain, respectively, with no perceivable dependence on the recoveries as a function of analyte concentration. The extraction recovery for the I.S. was great-

er than 88% in all instances. Typical chromatograms after extraction of fortified serum, urine and brain standards along with a serum blank are shown in Fig. 2.

Linearity and sensitivity

Linear calibration graphs were obtained over the concentration ranges 5 ng/ml–20 μg/ml, 20 ng/ml–20 μg/ml, and 50 ng/g–200 μg/g, for serum, urine and brain, respectively, with correlation coefficients greater than 0.999 and intercepts not significantly ($p > 0.05$) different from zero. The linear regression equations were $y = (0.1239 \pm 0.0026)x - (0.0309 \pm 0.0132)$, $y = (0.1270 \pm 0.0018)x - (0.0364 \pm 0.0240)$ and $y = (0.1301 \pm 0.0032)x - (0.0781 \pm 0.0792)$ ($n = 4$) for serum, urine and brain standard curves, respectively, where y is the peak-height ratio of drug to I.S. and x is the drug concentration. The LOQ at which the precision (R.S.D.) and accuracy (bias) were acceptable (<20%) were 5 and 20 ng/ml for serum and urine, respectively, and 50 ng/g for brain, based on using 1 ml of serum or urine and 200 mg of brain for extraction.

Precision and accuracy

As listed in Table I, intra-assay ($n = 15$) and inter-assay ($n = 12$) precisions ranging from 3.5 to 8.9%, 3.6 to 8.6% and 3.3 to 11.7%, for serum, urine and brain, respectively, were obtained at the three concentrations studied. The accuracy was ⩽10.0% in all instances. The system precision, determined by injecting a prepared sample five times, was found to be not greater than 0.8% in most instances.

Application

The proposed method was applied to the determination of U-82217 concentrations in serum and brain samples collected from a pilot pharmacokinetic study in rats. The study was conducted using 66 male Sprague–Dawley rats with approximate masses of 250 g. The rats were randomized into four dose groups using body masses. The vehicle group, consisting of three rats, received a single oral dose of control article and were killed for blood sampling 1 h after dosing. The remaining three groups, each containing 21 rats, received a single oral dose of U-82217 at levels of 10, 30 or 100 mg/kg. The control article (vehicle: 98.35% purified water, 1.25% Avicel 591, 0.2% sorbic acid and 0.2% polysorbate) and the U-82217 suspension formulation were administrated orally by gastric intubation. Three rats were killed for blood and brain sampling at each of 0.5, 1, 2, 4, 8, 16, and 24 h after dosing. The blood samples were allowed to clot at room temperature

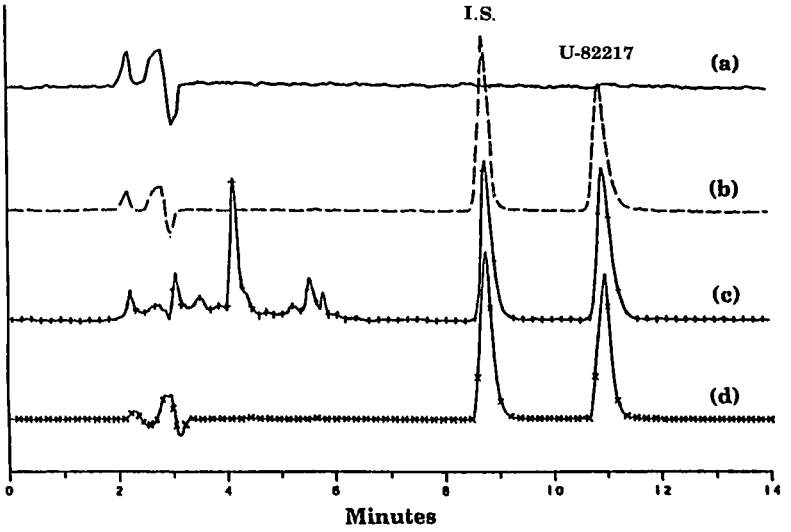


Fig. 2. Typical chromatograms after extraction of (a) rat serum blank, (b) rat serum blank fortified with 0.5 μg/ml each of U-82217 and I.S., (c) rat urine blank fortified with 1 μg/ml each of U-82217 and I.S. and (d) rat brain sample fortified with 5 μg/g of U-82217 and 0.5 μg/ml of I.S. Detection wavelength, 318 nm.

TABLE I

INTRA- AND INTER-ASSAY PRECISION AND ACCURACY

Sample	Theoretical concentration	Intra-assay (n = 5)		Inter-assay (n = 4)	
		Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Serum	10 ng/ml	7.4	8.0	6.8	10.0
	500 ng/ml	3.5	1.6	4.0	−4.4
	10 μg/ml	3.9	2.0	8.9	1.0
Urine	20 ng/ml	8.6	−1.5	10.4	5.5
	500 ng/ml	4.4	−4.2	3.6	−4.9
	10 μg/ml	3.8	5.0	5.2	−2.0
Brain	100 ng/ml	4.9	−1.6	11.7	4.2
	5 μg/ml	4.2	−4.0	8.1	−4.0
	100 μg/ml	5.0	3.2	3.3	−1.9

for 15–20 min and the serum was harvested by centrifugation, then transferred into a clean tube. Brain samples were frozen on dry-ice immediately and serum and brain samples were stored at −20°C until analysis.

The average serum concentration–time curves of each dose level for U-82217 are shown in Fig. 3. The low systemic serum concentration of U-82217 (<0.12 μg/ml for all doses) in rats might reflect poor absorption owing to the low water solubility of this drug (0.4 μg/ml). The concentration of U-82217 in brain at dose levels of 10 and 30 mg/kg were below the level of detection except for one brain sample that contained 52 ng/g of U-82217 at 2 h after a dose of 30 mg/kg. The brain concentrations were all less than 0.17 μg/g at a dose level of 100 mg/kg. These results agree with the U-82217 serum concentrations, which suggested poor oral bioavailability of U-82217 in rats.

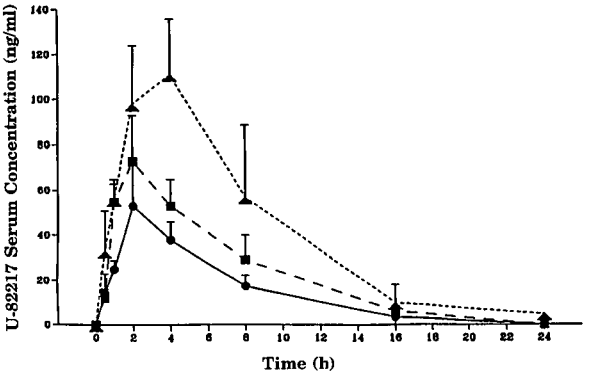


Fig. 3. Average serum concentration–time profiles of U-82217 in rats after single oral administration of (●) 10 mg/kg, (■) 30 and (▲) 100 mg/kg of U-82217.

ACKNOWLEDGEMENTS

The author thanks T.A. Jackson, D.M. Brussee and J.J. Cypher for collaboration in conducting the pharmacokinetic study with U-82217 in rats, G. E. Padbury for valuable comments on the manuscript and J.E. Katz for assistance with the preparation of the manuscript.

REFERENCES

1 K. C. VanHorne, (Editor), *Sorbent Extraction Technology*, Analytichem International, Harbor City, CA, 1985, p. 110.
2 P. F. VonVoigtlander, personal communication.
3 C. V. Puglisi, J. Pao, F. J. Ferrara and J. A. F. de Silva, *J. Chromatogr.*, 344 (1985) 199.
4 C. V. Puglisi, J. Pao, F. J. Ferrara and I. Bekersky, *J. Chromatogr.*, 417 (1987) 347.
5 U. Timm and M. Zell, *Arzneim.-Forsch.*, 33 (1983) 358.
6 C. D. Syracuse, B. R. Kuhnert, C. J. Kaine, A. C. Santos and M. Finster, *J. Chromatogr.*, 380 (1987) 145.
7 P. Heizmann, J.-C. Jordan and B. Ludvig, *J. Chromatogr.*, 415 (1987) 170.
8 V. P. Shah, K. K. Midha, S. Dighe, I. J. McGilveray, J. P. Skelly, A. Yacobi, T. Layloff, C. T. Viswanathan, C. E. Cook, R. D. McDowall, K. A. Pittman, and S. Spector, *J. Pharm. Sci.*, 81 (1992) 309 .

Journal of Chromatography, 629 (1993) 89–93
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 508

High-performance liquid chromatographic method for the determination of dimethindene in urine

D. Chollet, P. Künstner and M. Wermeille

Department of Toxicology and Pharmacokinetics, Zyma SA, CH-1260 Nyon (Switzerland)

ABSTRACT

An automated high-performance liquid chromatographic assay, using on-line solid-phase extraction, is described for the determination of dimethindene in urine. The solid-phase extraction of the sample (1000 μl) and the elution of the drug on to the analytical column are performed automatically and concomitantly. The limit of quantification is 5 pmol/ml.

INTRODUCTION

When a drug is applied topically it can cross the skin and reach the systemic circulation. Concentrations of the drug in the blood can therefore give a measure of its percutaneous absorption. The skin is known to be an excellent barrier to the permeation of substances and therefore the amount of a drug found in the blood is often very low. Hence, sensitive analytical methods able to determine the drug are needed.

An enzyme-linked immunosorbent assay (ELISA) has been found to be useful for the determination of the antihistaminic drug dimethindene maleate, N,N-dimethyl-3-[1-(2-pyridinyl)ethyl)]-1*H*-indene-2-ethanamine maleate, in serum at concentrations as low as 0.5 pmol/ml following oral and intravenous administration [1]. For topical administration, serum levels are very low and the ELISA technique is not sensitive enough. However, concentrations of dimethindene found in urine are higher than in the blood and can be determined using high-performance liquid chromatography (HPLC) [2] and gas chromatography [3]. The chromatographic methods were suitable for its determination at the 10 pmol/ml level in urine. However, they involve a tedious liquid–liquid extraction and evaporation–redissolution. In addition, they require deactivation of the glassware with silylating reagents to limit the strong adsorption of dimethindene on the glass. Unfortunately, ELISA cannot be used to determine dimethindene in urine owing to cross-reactions.

Application of automated on-line solid-phase extraction to biopharmaceutical analysis has been shown to be effective [4–7]. In this paper, a fully automated on-line HPLC system, using solid-phase extraction with automatic extraction cartridge exchange, is described.

EXPERIMENTAL

Chemicals

Anhydrous sodium acetate, ammonium sulphate, glacial acetic acid, fuming hydrochloric acid (37%) and sodium hydroxide, all of analytical-reagent grade, were purchased from Merck (Darmstadt, Germany). Ammonium acetate (BioChemika MicroSelect, >99%) was obtained from Fluka (Buchs, Switzerland). HPLC grade methanol and acetonitrile were from Mächler (Basle, Switzerland). Water was doubly distilled.

Dimethindene maleate (mol. mass 408.50) and its metabolite, N-desmethyldimethindene, were ob-

Correspondence to: D. Chollet, Department of Toxicology and Pharmacokinetics, Zyma SA, CH-1260 Nyon, Switzerland.

0021-9673/93/$06.00

tained from Zyma (Nyon, Switzerland). All reference solutions were freshly prepared in drug-free urine. A reference stock solution was prepared by dissolving about 20 mg (49 μmol) of either dimethindene maleate or its metabolite in 10.0 ml. Dilute solutions were obtained by successive dilution with the drug-free urine used for the reference solutions.

Apparatus

The chromatographic system consisted of three Model 6000A pumps, a Model 680 gradient/event controller and a Model 710B intelligent sample processor (WISP), all from Waters Assoc. (Milford, MA, USA). A Model OSP-2 on-line sample preparator was obtained from Merck. A Model 783A variable-wavelength UV detector was obtained from Applied Biosystems (Foster City, CA, USA). The WISP was equipped with a 2000-μl fixed loop, a 2.5-ml syringe and a cooling unit set to 10°C. Full details of the system configuration have been described in a previous paper [5].

LiChroCART cartridges (4 mm × 4.0 mm I.D.) from Merck, which were packed with either LiChrospher 100 CN (particle diameter, d_p = 5 μm) or LiChrospher 100 RP-18 (d_p = 5 μm), were used for on-line solid-phase extraction. The analytical column (Suplex pKb-100, d_p = 5 μm) (150 mm × 4.6 mm I.D.) was purchased from Supelco (Bellefonte, PA, USA).

Data acquisition and integration were performed using a Maxima 820 datastation from Waters Assoc.

Chromatography

Extraction and elution on to the analytical column were performed concomitantly by the OSP-2 on-line sample preparator, which was activated by the gradient/event controller following the instructions in Table I.

The HPLC mobile phases used were as follows: (A) mixture of 0.01 *M* sodium acetate and 0.04 *M* ammonium sulphate, adjusted to pH 3.0 with acetic acid–acetonitrile (90:10, v/v); (B) solution of 0.05 *M* ammonium acetate buffer adjusted to pH 7.0 with 1 *M* sodium hydroxide; (C) methanol–water (90:10, v/v). All aqueous solutions used were filtered on Zetapor membranes (pore size 0.22 mm) from Cuno (Meriden, CT, USA). The mobile phases were quickly degassed *in vacuo* prior to use. The flow-rates of the pumps were varied according to the instructions in Table II. The injection volume for samples and standards was 1000 μl. Detection was performed at 258 nm. The run time and the acquisition time were set to 28 min. Typical chromatograms are given in Fig. 1.

TABLE I

EVENT SETTINGS OF THE GRADIENT/EVENT CONTROLLER FOR ON-LINE SAMPLE PREPARATOR ACTIVATION

Time (min)	Event	Action	Comment
0.00	1	On	Valve 1 in position 2
0.00	3	On	Clamp closed (ready for extraction)
0.00	5	Off	Move direction set (left to right)
4.50	1	Off	Valve 1 in position 1(bypass of extraction side, end of washing)
4.60	3	Off	Clamp opened
4.70	4	Pulse	Move (1 cartridge from extraction side to analytical side)
4.80	3	On	Clamp closed (ready for analysis)
5.00	2	On	Valve 2 in position 2 (desorption, start of drug elution)
5.50	1	On	Valve 1 in position 2 (conditioning of a new cartridge)
14.00	2	Off	Bypass of valve 2 (initial setting)

Sample preparation

In order to prevent drug adsorption, the 4-ml WISP vials were directly filled from the 2-l flasks usually used for urine collection in pharmacokinetic studies, without any pipetting device.

TABLE II

FLOW-RATE SETTINGS OF THE GRADIENT/EVENT CONTROLLER

Time (min)	Flow-rate (ml/min)	Eluent A (%)	Eluent B (%)	Eluent C (%)	Curve[a]
Initial	2.20	45	0	55	–
6.00	3.20	62	0	38	11
8.00	3.20	0	62	38	11
14.00	3.20	62	0	38	11
20.00	2.20	45	0	55	11
35.00	0.00	45	0	55	11

[a] Curve 11 : new values are instantaneously set without any flow or eluent gradient.

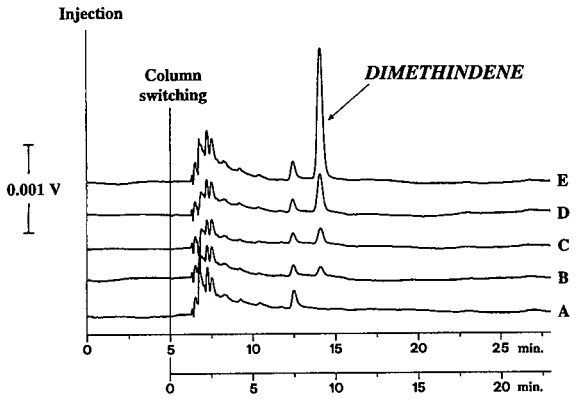


Fig. 1. Typical chromatograms of urine standards: (A) 0.0; (B) 4.9; (C) 12.3; (D) 24.5; (E) 49.0 pmol/ml. For experimental conditions, see text.

Standard preparation

Standard urine samples were prepared by diluting drug-free urine with reference solutions.

Calibration and quantification

Calibration was performed by linear regression analysis of the detector response over the concentration range 4.9–49.0 pmol/ml of dimethindene. The concentration of dimethindene equivalent in each sample (pmol/ml) was calculated using the calibration graphs.

Method validation

The recovery was determined according to ref. 8. The linearity range was checked by the correlation coefficient (r) of a calibration graph in the range 4.9–490 pmol/ml. The linearity of the calibration graphs was checked by their correlation coefficient in the range 4.9–49.0 pmol/ml. The precision was determined in terms of repeatability and reproducibility (n = 6). The accuracy was determined as the percentage deviation between the mean concentration found (n = 6) and the theoretical concentration. The stability of dimethindene under the experimental conditions was investigated.

RESULTS AND DISCUSSION

Solid-phase extraction

Systematic investigations were carried out combining either a cyanopropyl (CN) or an octadecyl (RP-18) reversed-phase extraction cartridge with a CN or a deactivated reversed-phase (Suplex pKb-100) analytical column. The combination of a CN cartridge and a Suplex analytical column was found to give more reproducible results than the other combinations.

Dimethindene could not be extracted onto the short CN cartridges in its fully protonated form, *i.e.*, in acidic medium. However, good extraction efficiency was observed with spiked urine samples using ammonium acetate buffer (pH 7.0). The recovery of dimethindene was found to be higher than 100% (n = 4) for a standard 49.0 pmol/ml urine. This result demonstrated that dimethindene in aqueous solutions (pH 7) is strongly adsorbed on plastic and glassware, as suggested by others [2,3]. Investigations of the adsorption of dimethindene on glass were carried out with dimethindene maleate standards in water and urine. The results showed that, in contrast to water, urine prevented this adsorption. Therefore, all the reference and standard solutions were prepared in drug-free urine.

As shown in Fig. 2, very efficient clean-up was obtained. Almost all of the matrix components were washed out of the cartridge within 4.5 min (see also Table I).

The extraction capacity of the cartridges was found to be higher than 490 pmol/ml. The plot of dimethindene peak height *versus* its concentration in prepared standards was linear in the range 4.9–490 pmol/ml (r = 1.000).

The relative standard deviation for the cartridge-to-cartridge variation of the dimethindene peak height was found to be 1.3% for a standard 49.0

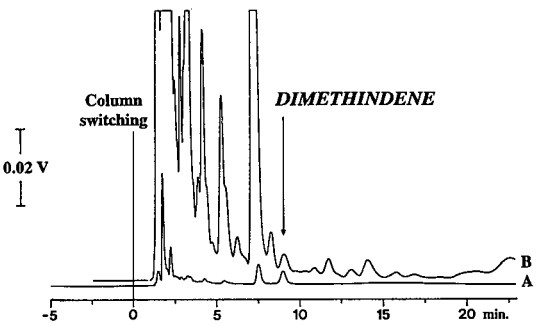


Fig. 2. Clean-up of the same urine sample under the experimental conditions with a washing time of (A) 4.5 and (B) 2.0 min. The time needed for a cartridge move was 0.5 min. For experimental conditions, see text.

TABLE III

PRECISION AND ACCURACY OF DIMETHINDENE ASSAY

Analyses carried out under the described experimental conditions.

Nominal concentration (pmol/ml)	Concentration found (mean ± S.D., n = 6) (pmol/ml)	R.S.D.[a] (%)	Confidence interval of the mean value (P = 95%) (pmol/ml)	Accuracy[b] (%)
Intra-assay variability				
4.9	4.76 ± 0.29	6.0	4.76 ± 0.30	−2.9
12.3	12.21 ± 0.63	5.1	12.21 ± 0.66	−0.3
24.5	24.90 ± 0.88	3.5	24.90 ± 0.93	1.6
49.0	48.89 ± 2.43	5.0	48.89 ± 2.55	−0.3
Inter-assay variability				
4.9	4.82 ± 0.46	9.6	4.82 ± 0.49	−1.7
12.3	12.28 ± 0.78	6.4	12.28 ± 0.82	0.2
24.5	24.89 ± 1.82	7.3	24.89 ± 1.91	1.6
49.0	49.10 ± 1.14	2.3	49.10 ± 1.20	0.2

[a] Relative standard deviation.

[b] Defined as the percentage deviation between the mean concentration found and the theoretical concentration.

pmol/ml urine sample (n = 10). No peak broadening was observed. One cartridge could be used up to 24 times.

Chromatography

Dimethindene was desorbed from the cartridge and eluted onto a deactivated reversed-phase analytical column (Suplex pKb-100) by isocratic elution with a mobile phase consisting of buffers (pH 3.0)–acetonitrile (90:10, v/v). This column was especially designed for the determination of basic compounds such as antihistamines. The dimethindene metabolite N-desmethyldimethindene [2] eluted at the same retention time as dimethindene. The UV spectra of dimethindene and its metabolite, recorded in solution in the mobile phase, showed that their molar absorptivities are identical. As the objective of the study was to determine the amount of drug reaching the systemic circulation, the measured amount of dimethindene included dimethindene plus N-desmethyldimethindene. Under the described experimental conditions, the recoveries of dimethindene and its metabolite were identical.

N-Desmethyldimethindene could be separated from dimethindene ($\alpha_{i,\text{dimethindene}}$ = 0.78) using 0.05 M ammonium acetate buffer (pH 7.0)–acetonitrile (77:23, v/v) as the mobile phase, but variations in the N-desmethyldimethindene peak area of 30% were observed. These variations were considered to be unacceptable. In a separate study [9], the concentration of the metabolite in the urine of healthy volunteers, collected after topical administration of dimethindene maleate, was found to be *ca.* 20% of the dimethindene content.

Method validation

The precision and the accuracy were determined over the range 4.9 - 49 pmol/ml. The results (Table III) ranged from 2.3 to 9.6% and from −1.7 to 1.6%, respectively. The parameters of the linear regressed calibration graphs, relating the dimethindene peak height to its concentration in prepared standards, were typically slope = 0.0343 and intercept = 0.26 with a correlation coefficient (r) of 0.999. The linearity range was from 4.9 to 490 pmol/ml of urine (r = 1.000). The limit of quantification for the assay was of the order of 5 pmol/ml. Dimethindene was found to be stable under the experimental conditions for up to 12 h. The relative standard deviation measured for a standard sample of 24.5 pmol/ml analysed every 30 min was 3.9% (n = 23). No drift of peak height was observed.

CONCLUSION

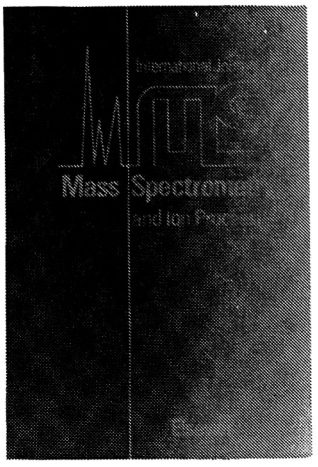
The method developed is suitable for the determi-

nation of dimethindene in urine at the pmol/ml level. It allows fully automated extraction and analysis of urine samples. Detailed validation data demonstrate its reliability.

REFERENCES

1 V. Arnera, M. Wermeille, M. Wellman, J. B. Llull, M. A. Althaus and L. P. Balant, *Arzneim.-Forsch.*, 40 (1990) 1346.
2 S. Radler and G. Blaschke, *J. Chromatogr.*, 567 (1991) 229.
3 M. Wermeille and G. A. Huber, *J. Chromatogr.*, 228 (1982) 187.
4 R. D. McDowall, *J. Chromatogr.*, 492 (1989) 3.
5 D. Chollet and M. Salanon, *J. Chromatogr.*, 593 (1992) 73.
6 D. Chollet and M. Wermeille, *Chimia*, 4 (1992).
7 D. Chollet and P. Künstner, *J. Chromatogr.*, 577 (1992) 335.
8 R. Huber and K. Zech, in R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in HPLC, Part A*, (*Journal of Chromatography Library*, Vol. 39A), Elsevier, Amsterdam, 1988, p. 126.
9 M. Salanon, Zyma, Nyon, personal communication, 1992.

END OF SYMPOSIUM PAPERS



Audience

All Mass Spectrometrists and those interested in the chemistry of charged particles.

PUBLICATION TIME ≤ 16 WEEKS!

INTERNATIONAL JOURNAL OF MASS SPECTROMETRY AND ION PROCESSES

AIMS AND SCOPE

The journal contains papers dealing with fundamental aspects of mass spectrometry and ion processes, and the study of the application of mass spectrometric techniques to specific problems. The following topics, amongst others, can be found:

- ❖ Theoretical and experimental studies of ion formation (i.e. by electrons, laser or other forms of radiation, heavy ions, high-energy particles, etc.), ion separation and ion detection processes.
- ❖ The design and performance of instruments (or their parts) and accessories.
- ❖ Measurements of natural isotopic abundances, precise isotopic masses, ionization, appearance and excitation energies, ionization cross-sections.
- ❖ Development of techniques related to determining molecular structures, geological age determination, studies of thermodynamic properties, chemical kinetics, surface phenomena, radiation chemistry, and chemical analyses.
- ❖ Theory of mass spectra, application of computer techniques to mass spectral data.
- ❖ Chemistry and physics of cluster ions.
- ❖ Spectroscopy of gaseous ions including studies related to interstellar chemistry.
- ❖ Mechanistic studies of unimolecular processes and ion/molecule reactions in the gas phase including computational aspects, ion trajectory calculations, quantum mechanical studies of potential energy surfaces.
- ❖ Physical organic chemistry of isolated ions.
- ❖ Biological aspects of mass spectrometry.

A feature of the journal is the **Rapid Communications** - short articles of 4-5 printed pages, the content of which is of such urgency or importance to others working on the same subject that very rapid publication is required.

ABSTRACTED/INDEXED IN: Chemical Abstracts, Current Contents: Physical, Chemical & Earth Sciences, Excerpta Medica, INSPEC, Mass Spectrometry Bulletin, PASCAL/CNRS.

1993 SUBSCRIPTION INFORMATION

Volumes 118-128 (in 33 issues)

Dfl. 3938.00 / US $ 2372.50 (including postage)

ISSN 0168-1176

I would like ☐ a free sample copy of International Journal of Mass Spectrometry and Ion Processes.

☐ Instructions to Authors.

☐ to enter a subscription for 1993. Please send me a Proforma Invoice.

Name _____

Address _____

_____

_____

Elsevier Science Publishers
Attn. Eugene P.M. Wijnhoven
P.O. Box 330, 1000 AH Amsterdam
The Netherlands
Fax: (+31-20) 5862 845

In the USA & Canada
Attn. Judy Weislogel
P.O. Box 945, Madison Square Station
New York, NY 10160-0757, USA
Fax: (212) 633 3880

Atomic Absorption Spectrometry

Theory, Design and Applications

edited by **S.J. Haswell**, *School of Chemistry, University of Hull, Hull, UK*

Analytical Spectroscopy Library Volume 5

Atomic absorption spectroscopy is now a well-established technique for the determination of trace elements covering a wide range of analyte types. The early theory and instrumentation chapters incorporate recent trends in instrumental design and methodology, in particular those associated with electrothermal techniques and background correction. The major thrust of the book is represented by 14 application chapters which give an extensive well referenced review of the practical use of the technique written by experts drawn from their own speciality areas. These include the determination of trace elements in areas as diverse as environmental, chemical and industrial analysis.

Whilst the book is primarily concerned with atomic absorption spectroscopy, any analyst involved in sample handling prior to trace elemental analysis will find this book a valuable compendium of methodology drawn from a very wide range of applications. For the current user of the technique the well referenced sections critically evaluate the state-of-the-art, while for the newer user the text will form the basis of a good laboratory handbook which offers a comprehensive instruction on the theory and instrumental design in atomic absorption spectroscopy.

Contents:

1992 xx + 530 pages
Price: US $ 177.00 / Dfl. 345.00
ISBN 0-444-88217-0

Elsevier Science Publishers

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

PUBLICATION SCHEDULE FOR THE 1993 SUBSCRIPTION

Journal of Chromatography and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1992	N 1992	D 1992	J	F	
Journal of Chromatography	623/1 623/2 624/1 + 2	625/1 625/2	626/1 626/2 627/1 + 2	628/1 628/2 629/1 629/2	630/1 + 2 631/1 + 2 632/1 + 2 633/1 + 2	The publication schedule for further issues will be published later
Cumulative Indexes, Vols. 601–650						
Bibliography Section						
Biomedical Applications				612/1	612/2	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 609, pp. 439–445. A free reprint can be obtained by application to the publisher, Elsevier Science Publishers B.V., P.O. Box 330, 1000 AH Amsterdam, The Netherlands.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Review articles, Short Communications and Discussions. Short Communications are usually descriptions of short investigations, or they can report minor technical improvements of previously published procedures; they reflect the same quality of research as Full-length papers, but should preferably not exceed five printed pages. Discussions (one or two pages) should explain, amplify, correct or otherwise comment substantively upon an article recently published in the journal. For Review articles, see inside front cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he/she is submitting the paper for publication in the *Journal of Chromatography*.

Manuscripts. Manuscripts should be typed in **double spacing** on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Abstract, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Abstract. All articles should have an abstract of 50–100 words which clearly and briefly indicates what is new, different and significant. No references should be given.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press" (journal should be specified), "submitted for publication" (journal should be specified), "in preparation" or "personal communication".

Dispatch. Before sending the manuscript to the Editor please check that the envelope contains four copies of the paper complete with references, legends and figures. One of the sets of figures must be the originals suitable for direct reproduction. Please also ensure that permission to publish has been obtained from your institute.

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.