JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

HPLC
TLC
Capillary Electrophoresis
Supercritical Fluid Techniques
Membrane Technology
Field-Flow Fractionation
Preparative & Analytical Separations

formerly Journal of Liquid Chromatography

VOLUME 19 NUMBER 4 1996

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

February 1996

Aims and Scope. The journal publishes an outstanding selection of critical, peer-reviewed papers dealing with analytical, preparative and process-scale liquid chromatography of all types and related technologies such as TLC; capillary electrophoresis; supercritical fluid extraction and chromatography; membrane separation technology; field-flow techniques; and others. As new separation technologies are introduced, they will also be included in the journal. On a regular basis, special topical issues are devoted to specific technologies and applications. Book reviews, software reviews and a calendar of meetings, symposia and expositions are also included.

Identification Statement. *Journal of Liquid Chromatography & Related Technologies* (ISSN: 1082-6076) is published semimonthly except monthly in May, August, October, and December for the institutional rate of $1,595.00 and the individual rate of $797.50 by Marcel Dekker, Inc., P.O. Box 5005, Monticello, NY 12701-5185. Second Class postage paid at Monticello, NY. POSTMASTER: Send address changes to *Journal of Liquid Chromatography & Related Technologies*, P.O. Box 5005, Monticello, NY 12701-5185.

Volume	Issues	Institutional Rate	Individual Professionals' and Student Rate	Foreign Postage		
				Surface	Airmail to Europe	Airmail to Asia
19	20	$1,595.00	$797.50	$70.00	$110.00	$130.00

Individual professionals' and student orders must be prepaid by personal check or may be charged to MasterCard, VISA, or American Express. Please mail payment with your order to: Marcel Dekker Journals, P.O. Box 5017, Monticello, New York 12701-5176.

CODEN: JLCTFC 19(4) i–iv, 509–686 (1996)
ISSN: 1082-6076

Printed in the U.S.A.

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography & Related Technologies* are selectively indexed or abstracted in:

■ Abstracts Journal of the Institute of Scientific and Technical Information of the Russian Academy of Sciences ■ Alerts ■ Aluminium Industry Abstracts ■ Analytical Abstracts ■ ASCA ■ Berichte Pathologie ■ CAB Abstracts ■ Cambridge Scientific Abstracts ■ Chemical Abstracts ■ Chemical Reactions Documentation Service ■ Current Awareness in Biological Sciences ■ Current Contents/Life Sciences ■ Current Contents/Physical and Chemical Sciences ■ Current Opinion ■ Engineered Materials Abstracts ■ Engineering Index ■ Excerpta Medica ■ Metals Abstracts ■ Reference Update ■ Saltykov-Shchedrin State Public Library ■ Science Citation Index ■ Tobacco Abstracts

Manuscript Preparation and Submission. See end of issue.

This journal is also available on CD-ROM through ADONIS™ beginning with the 1991 volume year. For information contact: ADONIS, Marketing Services, P.O. Box 17005, 1001 JA Amsterdam, The Netherlands, Tel: +31-20-626-2629, Fax: +31-20-626-1437.

The journals of Marcel Dekker, Inc. are available in microform from: University Microfilms, Inc., 300 North Zeeb Road, Ann Arbor, Michigan 48106-1346, Telephone: 800-521-0600; Fax: (313) 761-1203.

Contributions to this journal are published free of charge.

Effective with Volume 6, Number 11, this journal is printed on acid-free paper.

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 509–520 (1996)

NORMAL PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TOCOPHEROLS ON POLAR PHASES

S. L. Abidi* and T. L. Mounts

Food Quality and Safety Research
USDA-ARS-NCAUR
1815 North University Street
Peoria, IL 61604 USA.

ABSTRACT

Five plant tocopherols were separated by normal phase high performance liquid chromatography (HPLC) on aminopropylsilica or diol-bonded silica with fluorescence detection. HPLC characteristics of these compounds were studied under various mobile phase conditions. Mobile phases employed binary solvent systems comprising a hydrocarbon and an alcohol, an ether or an ester. Separation factors (α) of adjacent ring-methylated tocol components were determined.

Employment of aprotic weakly polar modifiers in the mobile phases increased the α values for the pair of 5,8- and 7,8-dimethyltocol, but decreased those for the 5,7-and 5,8-dimethyltocol pair. Both component resolution and detection sensitivity were adversely affected by the use of a diol-bonded silica phase of large particle size. HPLC with cyclohexane-binary eluents invariably led to lower values of analyte capacity factors (k') than with hexane counterparts. Further, regardless of the type of column evaluated, the tocol isomers eluted in an ethereal mobile phase in the order of increasing k' values:

k'(tetrahydrofuran) <k'(dioxane) < k'(t-butyl methyl ether < k'(diisopropyl ether). In HPLC with protic co-solvents, the observed differences in k' values among the three dimetyltocols were interpreted in terms of methyl substitution effects.

INTRODUCTION

Our continuous interest in distribution patterns of antioxidants in oil seeds has prompted current methodological studies on the separation and quantitation of tocopherol isomers in plant oils. Mobile phase solvent systems employed in the normal phase high-performance liquid chromatographic (HPLC) separation of β- and γ-tocopherols are critical determinants for the accurate analysis of the antioxidant pair at trace levels. To understand factors influencing the separation of closely related tocopherols, comprehensive evaluation of HPLC methods with solvents of variable polarity is of analytical importance.

Complete reverse phase HPLC separations of all soybean tocopherol isomers are difficult. However, normal phase HPLC resolution of the compounds has met with much success. The majority of published methods employed silica columns.[1-13] In contrast, there are few reports in the literature describing the use of polar silica-based columns.[14-16] A recent study[14] showed that normal phase elution characteristics of methylated tocol isomers on cyclodextrin-bonded silica (CDS) were partly dictated by the substitution patterns of the 2-methyl-6-chromanol ring system. As an extension of the previous study, a similar approach was taken to examine the structural effects on the HPLC behavior of the title compounds on different polar phases. The results are reported in this paper.

EXPERIMENTAL

Materials

Methyl substituted tocol standards (Figure 1) α-tocopherol (5,7,8-trimethyltocol) (TMT), β-tocopherol (5,8-dimethyltocol) (DBT), γ-tocopherol (7,8-dimethyltocol) (DGT), 5,7-dimethyltocol (DMT), and L-tocopherol (8-methyltocol) (MDT) were obtained from Matreya, Inc. (Pleasant Gap, PA, U.S.A.). All solvents were HPLC- grade and were used as supplied. Hexane (HX), cyclohexane (CHX), dioxane (DIOX), tetrahydrofuran (THF), and ethyl acetate (ETAC) were obtained from Fisher Chemicals (Fair Lawn, NJ, U.S.A.). Other solvents 1-propanol (1-PR), 2-propanol (2-PR), t-butyl methyl ether

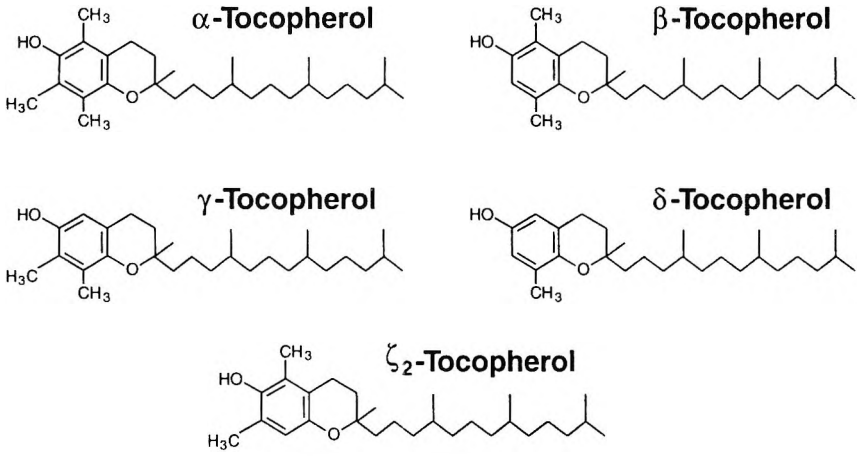


Figure 1. Structures of soybean tocopherols and 5,7-dimethyltocol (ζ_2-tocopherol).

(TBM), and diisopropyl ether (IPIP) were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Methods

All HPLC experiments were performed on a Thermo Separation Products (formerly Spectra-Physics, San Jose, CA, U.S.A.) liquid chromatograph Model SP8700 solvent delivery unit. An Applied Biosystems (Foster City, CA, U.S.A.) Model 980 programmable fluorescence detector was interfaced with the LC system to monitor column effluents. The detector was set at an excitation wavelength of 298 nm and an emission wavelength of 345 nm. Mobile phases were prepared by mixing hexane or cyclohexane with one of several oxygen-containing solvents (an alcohol, an ether, or an ester) at variable proportions to form binary solvent systems.

Samples containing tocol isomers in hexane (100 μg/mL) were injected onto a column via an Applied Biosystems silica guard column (15 x 3.2 mm I.D.) and a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector fitted with a 10-μL loop. Before HPLC assays, all analytical samples were freshly prepared from individual tocol stock solutions which had been stored in amber vials in a freezer. Capacity factors ($k' = t/t_0 - 1$) were determined for each tocol component, where t and t_0 represent average retention times based on three

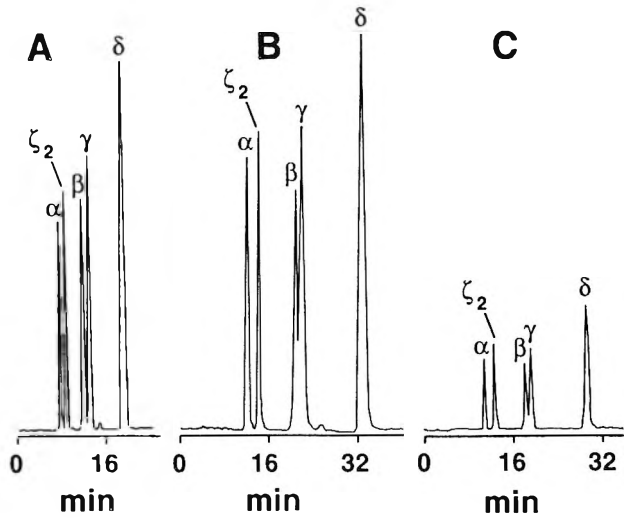


Figure 2. Separations of tocopherols with strongly polar modifiers. Columns: (A) amino-Si, (B) diol-Si(10 μm), (C) diol-Si(5 μm). Mobile phases: (A) hexane-dioxane(90:10), (B) hexane-dioxane (95:5), (C) cyclohexane-dioxane (97:3).

replicate determinations of an analyte and an unretained solute, respectively. Separation factors ($\alpha = k'_{i+1} / k'_i$) were obtained for adjacent components, where "i" represents a tocol isomer analyzed.

Stationary phases used in this study include (1) Waters μ-Bondapak NH_2 (aminopropylmethylsilyl bonded silica, 10 μm, 300 x 3.9 mm I.D.) (Milford, MA, U.S.A.), (2) Alltech Lichrosorb DIOL (1,2-hydroxy-3-propoxypropylsilyl bonded silica, 10 μm, 250 x 4.6 mm I.D.) (Deerfield, IL, U.S.A.), and (3) ES Industries Chromega Diol (1,2-hydroxypropylsilyl bonded silica, 5 μm, 250 x 4.6 mm I.D.) (Berlin, NJ, U.S.A.). Mobile phases were filtered, degassed, and pumped through the column at a flow rate of 1 mL/min. Prior to sample injections, it normally required 30 min to 1 hr to equilibrate a column.

RESULTS AND DISCUSSION

Normal phase HPLC results obtained with 10 μm-amino-, 10 μm-diol-, and 5 μm-diolsilica columns are summarized in Tables 1 and 2, Tables 3 and 4, and Tables 5 and 6, respectively. The three stationary phases were evaluated

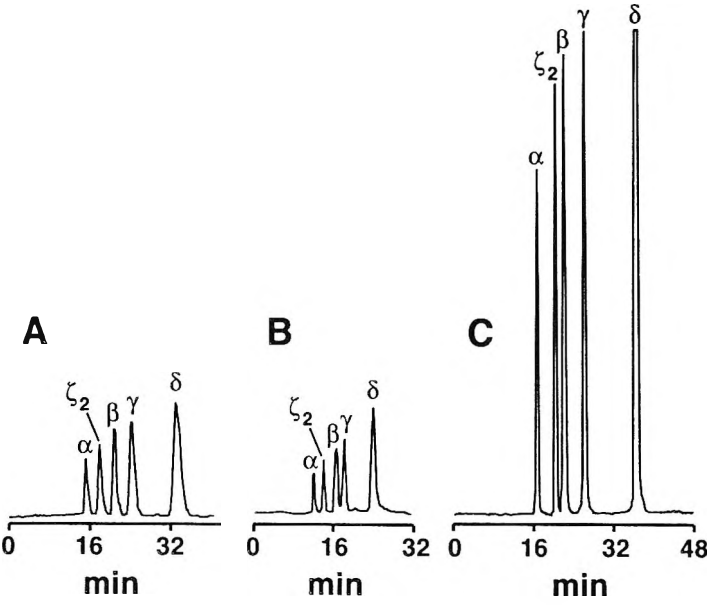


Figure 3. Separations of tocopherols with weakly polar modifiers. Columns: same as in Figure 2. Mobile phases: (A) cyclohexane-t-butyl methyl ether(90:10), (B) hexane-t-butyl methyl ether(90:10), (C) hexane-diisopropyl ether(90:10).

individually in conjunction with two hydrocarbon (cyclohexane and hexane)-binary mobile phase systems each of which contained seven different modifiers.

Examples of representative chromatograms obtained under various HPLC conditions are shown in Figures 2 and 3. Examination of the HPLC chromatograms revealed that mobile phase effects on the elution patterns were profound despite little influence of the polar stationary phases on the chromatographic profiles. Retention time differences (Δt) of β-ζ pairs in HPLC with dioxane as co-solvent were generally greater than those of γ-β pairs [i.e., $\Delta t(\beta\text{-}\zeta) > \Delta t(\gamma\text{-}\beta)$] (Figure 2). With mono-oxygenated ether as co-solvent, however, the magnitude of $\Delta t(\beta\text{-}\zeta)$ approached and became smaller than that of $\Delta t(\gamma\text{-}\beta)$ (Figure 3).

For analyses of tocopherols in soybean oils in which the least abundant β-isomer normally coexists with the most abundant γ-isomer, HPLC separations showing evenly dispersed tocol components such as those shown in Figure 3

should be the preferred methods of choice to ensure precise quantitation of the trace components in soybean oil samples. Based on our experience with canola oil assays, application of the methods with the weakly polar modifiers in mobile phases (e.g. Figure 3) to the analysis of canola tocopherols should facilitate confirmation of the absence of the β-isomer in the oils. With strongly polar modifiers (e.g. Figure 2), HPLC of the canola oil samples often yields erroneous results because of the appearance a peak at the retention time of the β-isomer in the close proximity of the most abundant γ-isomer on HPLC chromatograms.

As depicted in Figure 1, structures of the five investigated compounds TMT, DMT, DBT, DGT, and MDT differ in the number and position of methyl groups on the aromatic ring of the tocol molecules. Regardless of the type of stationary phases employed, normal phase separations of tocopherols by the number of methyl substitution (monomethyl- *vs* dimethyl- *vs* trimethyltocol) were rather straightforward having α values in the range 1.27-1.75. In HPLC systems where polar modifiers (e.g. 1-, 2-propanol, or dioxane) were used in mobile phases (Figure 2), the two dimethyltocols DBT (β-tocopherol) and DGT (γ-tocopherol) were not as readily resolved (α = 1.00-1.10) as the analogous DMT-DBT pair (α = 1.22-2.10). The differences in the α values among the pairs of the dimethylated tocol compounds can be explained based on molecular polarity and steric factors involving the 5-, 7-, and 8 methyls and the 6-hydroxy group.[14] The two methyl groups in DGT (the γ-isomer) apparently confer a higher degree of disymmetry and polarity in the aromatic moiety than those in DBT (the β-isomer) or DMT (the ζ-isomer) (Figure 1). Thus, the polarity of the three dimethylated compounds in the series appears to be inversely related to their retention times (t) or k' values: t or k' (DMT) < t or k' (DBT) < t or k' (DGT).

Similar to earlier findings from studies[14] with CDS phases, HPLC with mobile phases containing an ester or a mono-functional ether produced well separated β- and γ-tocopherol components (DBT and DGT) (Figure 3) with α values ranging from 1.08 to 1.30 (Tables 1-6). These α values were significantly higher than those obtained with an alcohol- or a polar ether-co-solvent described earlier. In the latter alcohol mobile phase, the adjacent analyte components were separated with α values that decreased in the following order: α (DMT-DBT) > α (DGT-MDT) > α (TMT-DMT) > α (DBT-DGT). Elution with mobile phases containing weakly polar oxygenated modifiers tended to lower the α values for the (DMT-DBT)- and (DGT-MD)-pairs with a concurrent increase in the separation (higher α values) of the β-γ-(DBT-DGT)-pair. Further, as expected, the β-isomer was moderately better separated from the γ-isomer in hexane-binary solvents than in cyclohexane-binary eluents.

Table 1

Separations of Methyl Substituted Tocols on Aminosilica (10μm) with Hexane (HX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile Phase	Component TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.05	(1.34)	1.41	(2.09)	2.94	(1.10)	3.23	(1.75)	5.64
HX-1-PR(99:1)	1.17	(1.30)	1.52	(1.97)	3.00	(1.10)	3.29	(1.75)	5.76
HX-THF(90:10)	1.16	(1.28)	1.49	(1.38)	2.05	(1.23)	2.52	(1.52)	3.82
HX-DIOX(90:10)	1.35	(1.13)	1.52	(1.66)	2.52	(1.12)	2.82	(1.65)	4.64
HX-TBM(90:10)	5.35	(1.23	6.58	(1.22)	8.05	(1.30)	10.5	(1.44)	15.1
HX-IPIP(90:10)	9.70	(1.30)	12.6	(1.29)	16.3	(1.27)	20.7	(1.48)	30.3
HX-ETAC(90:10)	1.47	(1.28)	1.88	(1.44)	2.70	(1.26)	3.41	(1.53	5.23

For solvent and compound abbreviations, see EXPERIMENTAL.

Table 2

Separations of Methyl Substituted Tocols on Aminosilica (10μm) with Cyclohexane (CHX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile Phase	Component TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.00	(1.35)	1.35	(2.00)	2.70	(1.07)	2.88	(1.74)	5.00
HX-1-PR(99:1)	1.11	(1.33)	1.48	(1.96)	2.91	(1.06)	3.08	(1.73)	5.24
HX-THF(90:10)	1.03	(1.28)	1.32	(1.37)	1.80	(1.21)	2.18	(1.50)	3.27
HX-DIOL(90:10)	1.19	(1.14)	1.36	(1.60)	2.18	(1.11)	2.42	(1.63)	3.94
HX-TBM(90:10)	3.47	(1.24)	4.29	(1.22)	5.23	(1.19)	6.23	(1.40)	8.70
HX-IPIP(90:10)	7.61	(1.33)	10.1	(1.29)	13.1	(1.18)	15.4	(1.47)	22.6
HX-ETAC(90:10)	1.21	(1.27)	1.54	(1.50)	2.31	(1.23)	2.84	(1.49)	4.23

For solvent and compound abbreviations, see EXPERIMENTAL.

The retention data in Tables 1-6 indicate that tocol analytes were generally more strongly adsorbed (higher k' values) on a polar phase in 1-propanol than in 2-propanol. Surprisingly a reversal of this generalization was manifested in normal phase HPLC with a CDS phase.[14] All the tocol components exhibited strongest retention (highest k' values) on the stationary phase in a mobile phase system containing diisopropyl ether. In relation to the

open-chain ethers examined, cyclic ethers (e.g. tetrahydrofuran and dioxane) seemed to contribute to weak analyte adsorption on the column. Thus, the observed general trend of k' values of individual tocols in the presence of ether modifiers was as follows: k' (diisopropyl ether) > k' (t-butyl methyl ether) > k' (Dioxane) > k' (tetrahydrofuran).

Following a parallel approach, attempts at correlating retention characteristics (k' values) of individual tocol components listed in Tables 1-6 with all types of mobile phases of interest failed to delineate systematic orders of variations in k' values with the seven modifier varieties investigated. In all cases studied, the k' values observed in HPLC experiments with hexane-binary solvents were, as anticipated, higher than those obtained with the corresponding cyclohexane-systems. As to the differential mobile phase effects between the cyclic (cyclohexane) and straight-chain hydrocarbon (hexane) structures on the separation of tocopherols were minimal beside the observed polarity implications (Table 1 *vs* Table 2, Table 3 *vs* Table 4, and Table 5 *vs* Table 6).

A comparison of the HPLC data for the amino-column with the corresponding data obtained with diol-phases demonstrated that the selectivity of the amino column (Tables 1 and 2) for the β- and γ-components was somewhat superior (higher α values) to that of both the diol columns (5 μm and 10 μm) used (Tables 3-6). For a given tocol mixture, a highest degree of improvement in the separation of the β-γ pair was observed when a combination of an amino-column and hexane-t-butyl methyl ether (Table 1) was employed in HPLC experiments. To a less extent, enhancement in the resolution of the same pair in the mixture of tocols was found in experiments with the 5-μm-diol-column (Tables 5 and 6) and hexane (or cyclohexane)-t-butyl methyl ether.

Analysis of data obtained with the two diol-columns (Tables 3-6) showed that β- and γ-tocopherols were not separated (α = 1.00) on the 10-μm-diol phase when eluted with a cyclohexane-binary mobile phase containing strongly polar modifiers (alcohol or dioxane) (Table 4). In addition, HPLC with the latter 10-μm low-efficiency diol-column gave detector responses significantly inferior to those obtained with the corresponding 5-μm diol-column probably due to partial adsorption of analytes on the 10-μm column.

On the other hand, under identical mobile phase conditions in the presence of alcohol modifiers, the 10 μm-diol phase seemed to be more retentive than the amino-phase of the same particle size (10 μm) as reflected in the higher k'

Table 3

Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Hexane (HX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile	Component								
Phase	TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.44	(1.39)	2.00	(1.89)	3.77	(1.07)	4.05	(1.59)	6.44
HX-1-PR(99:1)	1.55	(1.36)	2.11	(1.90)	4.00	(1.07)	4.27	(1.64)	7.00
HX-THF(95:5)	2.00	(1.19)	2.38	(1.40)	3.33	(1.13)	3.77	(1.41)	5.33
HX-DIOX(95:5)	2.44	(1.23)	3.00	(1.63)	4.88	(1.07)	5.22	(1.58)	8.27
HX-TBM(95:5)	3.61	(1.26)	4.55	(1.46)	6.66	(1.12)	7.44	(1.49)	11.1
HX-IPIP(95:5)	5.27	(1.30)	6.83	(1.57)	10.7	(1.10)	11.8	(1.53	18.1
HX-ETAC(95:5)	2.33	(1.17)	2.72	(1.55)	4.22	(1.10)	4.66	(1.53)	7.11

For solvent and compound abbreviations, see EXPERIMENTAL.

Table 4

Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Cyclohexane (CHX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile	Component								
Phase	TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.27	(1.27)	1.61	(2.10)	3.38	(1.00)	3.38	(1.67)	5.66
HX-1-PR(99:1)	1.33	(1.33)	1.77	(2.13)	3.77	(1.00)	3.77	(1.71)	6.44
HX-THF(95:5)	1.52	(1.23)	1.88	(1.39)	2.61	(1.10)	2.88	(1.54)	4.44
HX-DIOX(95:5)	1.55	(1.25)	1.94	(1.66)	3.22	(1.00)	3.22	(1.64)	5.27
HX-TBM(95:5)	2.50	(1.26)	3.16	(1.44)	4.55	(1.10)	5.00	(1.57)	7.83
HX-IPIP(95:5)	3.33	(1.28)	4.27	(1.55)	6.61	(1.08)	7.16	(1.56)	11.2
HX-ETAC(95:5)	1.48	(1.24)	1.83	(1.51)	2.77	(1.10)	3.05	(1.53)	4.66

For solvent and compound abbreviations, see EXPERIMENTAL.

values of the tocol components on the diol phase (Table 1 vs Table 3; Table 2 *vs* Table 4). These particular observations in HPLC with the 10 μm-diol column were comparable to those with a β-CDS column (14) in which k' (β-CDS) > k' (aminosilica) presumably due to interactions between hydroxy groups in the diol- and CDS phases and the hydroxy group in analyte solutes during HPLC separation processes. The use of a relatively high efficiency 5 μm-diol column (such as the one used in this study) and a cyclohexane-binary

Table 5

Separations of Methyl Substituted Tocols on Diol-Bonded Silica (5 μm) with Hexane (HX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile	Component								
Phase	TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.44	(1.31)	1.88	(1.80)	3.38	(1.06)	3.58	(1.53)	5.48
HX-1-PR(99:1)	1.47	(1.35)	1.98	(1.89)	3.75	(1.07)	4.01	(1.62)	6.50
HX-THF(95:5)	2.00	(1.25)	2.50	(1.26)	3.16	(1.21)	3.83	(1.48)	5.66
HX-DIOX(95:5)	2.16	(1.21)	2.61	(1.47)	3.83	(1.10)	4.22	(1.51)	6.38
HX-TBM(95:5)	4.05	(1.27)	5.16	(1.21)	6.22	(1.21)	7.50	(1.48)	11.1
HX-IPIP(95:5)	6.33	(1.35)	8.55	(1.27)	10.9	(1.21)	13.2	(1.55)	20.4
HX-ETAC(95:5)	2.05	(1.27)	2.61	(1.36)	3.55	(1.20)	4.27	(1.51)	6.44

For solvent and compound abbreviations, see EXPERIMENTAL.

Table 6

Separations of Methyl Substituted Tocols on Diol-Bonded Silica (10 μm) with Cyclohexane (CHX) - Binary Mobile Phases

	HPLC Characteristics								
	k'	(α)	k'	(α)	k'	(α)	k'	(α)	k'
Mobile	Component								
Phase	TMT		DMT		DBT		DGT		MDT
HX-2-PR(99:1)	1.11	(1.30)	1.44	(1.69)	2.44	(1.07)	2.61	(1.57)	4.11
HX-1-PR(99:1)	1.16	(1.29)	1.50	(1.89)	2.83	(1.04)	2.94	(1.68)	4.94
HX-THF(95:5)	1.16	(1.15)	1.33	(1.29)	1.72	(1.16)	2.00	(1.50)	3.00
HX-DIOX(95:5)	1.33	(1.21)	1.61	(1.52)	2.44	(1.05)	2.55	(1.59)	4.05
HX-TBM(95:5)	2.33	(1.24)	2.88	(1.17)	3.38	(1.17)	3.94	(1.56)	6.16
HX-IPIP(95:5)	4.44	(1.30)	5.77	(1.18)	6.83	(1.15)	7.83	(1.57)	12.3
HX-ETAC(95:5)	1.32	(1.26)	1.66	(1.30)	2.16	(1.13)	2.44	(1.60)	3.83

For solvent and compound abbreviations, see EXPERIMENTAL.

solvent system was advantageous because rapid analyses were achieved without affecting component resolution.

In conclusion, the results of this study provide specific guidelines for the selection of HPLC columns and solvents in the separation of soybean tocopherols and 5,7-dimethyltocol which can be used as an internal standard for reliable quantitation purposes. The ortho-effect of two methyls on the

hydroxy group in tocopherols has significant bearing on separation patterns of components and can be optimized by adjusting mobile phase solvent polarity. In light of the ability of the hydrocarbon mobile phases modified with monofunctional ethers to achieve satisfactory dispersion of analyte components, they are highly recommended for use in the trace HPLC analysis of tocol compounds and related structures including tocotrienols.[17]

ACKNOWLEDGMENT

The authors thank Kathy A. Rennick for technical assistance.

REFERENCES

1. Y. L. Ha, A. S. Csallany, Lipids, **23**, 359 (1988).

2. W. D. Pocklington, A. Dieffenbacher, Pure Appl. Chem., **60**, 877 (1988).

3. A. Gapor, A. Kato, A. S. H. Ong, J. Am. Oil Chem. Soc., **63**, 330 (1986).

4. P. J. Vankerk, A. E. C. Burger, J. Am. Oil Chem. Soc., **62**, 531 (1985).

5. A. P. Carpenter, J. Am. Oil Chem. Soc., **56**, 668 (1979).

6. C. C. Tangney, J. A. Drisskell, H. M. McNair, J. Chromatogr., **172**, 513 (1979).

7. J. N. Thompson, G. Hatina, J. Liq. Chromatogr., **2**, 327 (1979).

8. G. T. Vatassery, V. R. Maynard, D. F. Hagen, J. Chromatogr., **161**, 299 (1978).

9. B. Nilsson, B. Johansson, L. Jansson, L. Holmberg, J. Chromatogr., **145**, 169 (1978).

10. G. T. Vatassery, D. F. Hagen, Anal. Biochem., **79**, 129 (1977).

11. M. Matsuo, Y. Tahara, Chem. Pharm. Bull., **25**, 3381 (1977).

12. C. D. Carr, Anal. Chem., **46**, 743 (1974).

13. P. J. Van Niekerk, Anal. Biochem., **52**, 533 (1973).

14. S. L. Abidi, T. L. Mounts, J. Chromatogr., **670**, 67 (1994).

15. W. Feldheim, H. Schult, R. Katerberg, Z. Lebensm.-Unters. Forsch., **178**, 115 (1984).

16. B. A. Ruggeri, T. R. Watkins, R. J. H. Gray, R. I. Tomlins, J. Chromatogr., **291**, 377 (1984).

17. S. L. Abidi, T. L. Mounts, unpublished results (1995).

Received September 24, 1995
Accepted October 4, 1995
Manuscript 3972

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 521–535 (1996)

ISOLATION AND COMPLETE SEPARATION OF LIPIDS FROM NATURAL SOURCES

Constantinos A. Demopoulos,[1] Smaragdi Antonopoulou,[1]
Nikolaos K. Andrikopoulos,[2] Vassilios M. Kapoulas[1]

[1]University of Athens
Department of Chemistry
Panepistimioupolis
15771, Athens, Greece
[2]Harokopio University of Home Economics
Department of Dietetics
70 El. Venizelou Street
17671' Athens. Greece

ABSTRACT

A method of isolation and complete separation of lipids from natural sources into classes and species is reported which combines our previously published techniques with new techniques described in this article for the first time. Pigments are separated from crude total lipid extracts with two successive TLC systems: a) petroleum ether/benzene/glacial acetic acid (30:70:2) and b) acetone/methanol/water (40:20:1). Pigment-free total lipids are separated on a silicic acid column into neutral, glyco- and phospholipids. Neutral, glyco- and phospholipids are separated into classes and species by suitable HPLC methods.

INTRODUCTION

The study of lipids has assumed considerable importance in recent years with the recognition that they are involved in many vital biological

processes in animals, plants and microorganisms. It is well known that lipids serve as a major storage form of energy and that they are responsible for maintaining the structural integrity of cells as the principal components of the membranes. Also, lipids are ingredients of all foods and their composition is obviously vital to good nutrition. Disturbances in lipid metabolism are known to accompany a variety of disease states and the role of lipids in heart disease remains an unresolved controversy. It has become apparent that certain lipids such as phosphatidylinositol and its metabolites are vital cellular messengers while the discovery of Platelet-Activating Factor,[1] an unusual species of phosphatidylcholine that possesses extremely potent biological activity, opened a new and unexpected chapter in the history of phospholipids.

Even though methods for the analysis of lipids are of great importance for many research, clinical and quality control applications and a lot of methods have been reported in the literature, to our knowledge the techniques provided are not appropriate for the complete separation of total lipids from natural sources.

Chromatography has been recognized as a useful separation technique in lipid analysis. Column chromatography (CC), thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) even though have served lipid analysis well over the years, do have a number of disadvantages such as poor recovery, doubtful reproducibility and unsatisfactory separation.

High performance liquid chromatography (HPLC) today offers considerable advantages as a qualitative and quantitative analytical tool. The wide range of column packing materials along with the variety of eluting systems available in HPLC permits successful separation of all the classes of lipids. In addition, resolution tends to fall off only slowly with increasing sample size, analysis time can be short, retention times of compounds under set conditions are reproducible in combination with the fact that the sample does not need to be derivatized and its lipid components are fully recovered (very important in the case where further biological and structural study has to be performed).

In the present study, a method of isolation and entire separation of lipids from natural sources into classes and species is reported which combines our previously published techniques with new techniques described in this article for the first time. These new techniques are a) a TLC system for the isolation of pigment-free total lipids b) a column chromatographic technique for separation of total lipids into neutral, glyco- and phospholipids and c) the separation of phospholipids into classes and species by HPLC. In the

discussion section some alternative techniques for the analysis of lipids are also presented which are already published.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, purchased from Merck (Darmstadt, G). HPLC solvents were purchased from Ruthburn (Walkerburn, Peebleshire, UK). Lipid standards of HPLC grade were obtained from Supelco (Bellefone, PA, USA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described.[1] Chromatographic material used for column chromatography was silicic acid 35-70 mesh ASTM 7733 (Merck, Darmstadt, G). Chromatoplates (20X20cm) were of analytical (0.25mm thickness) and preparative scale (1 mm thickness). Chromatographic material used for thin layer chromatography was silica gel G -Type 60 (Merck, Darmstadt, G). Blood samples were collected from patients with primary glomerular disease previously used for the determination of PAF levels in another study. Nettle (Urtica dioica) was collected from countryside (Attica, GR). Bovine brain was also used immediately after the sacrifice of the animal.

Preparation of Standards and Samples

All lipid standards were prepared as 5% solutions in chloroform/methanol (1:1). Homogenized nettle leaves and roots were extracted and the total lipids were obtained. Total lipids were extracted from blood, fractionated on silicic acid column chromatography and the fraction with the biologically active lipids was obtained.[2] This fraction was further separated in neutral and phospholipids with current counter distribution.[3] Total lipids were isolated from bovine brain by extraction.

Silicic Acid Column Preparation

The silicic acid, was washed with water and methanol and activated overnight at 120 °C. The glass column 13.5mm (I.D.), was slurry packed (46cm height) using chloroform. The lipid sample was dissolved in 0.5mL of chloroform/methanol (1: 1), then taken up with up to 2mL chloroform.

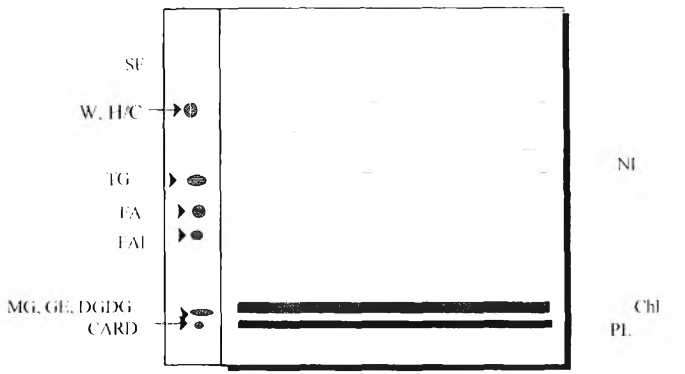


Figure 1. TLC development of standard lipids and of lipids from nettle in petroleum ether/benzene/glacial acetic acid (30:70:2).
SF: solvent front; W: waxes; HC: hydrocarbons; TG: triglycerides; FA: fatty acids; FAl: fatty alcohols; MG: monoglycerides; GE: glycerylethers; DGDG: digalactosyldiglycerides; CARD: cardiolipin; NL: neutral lipids; Chl: chlorophylles (pigments); PL: polar lipids.

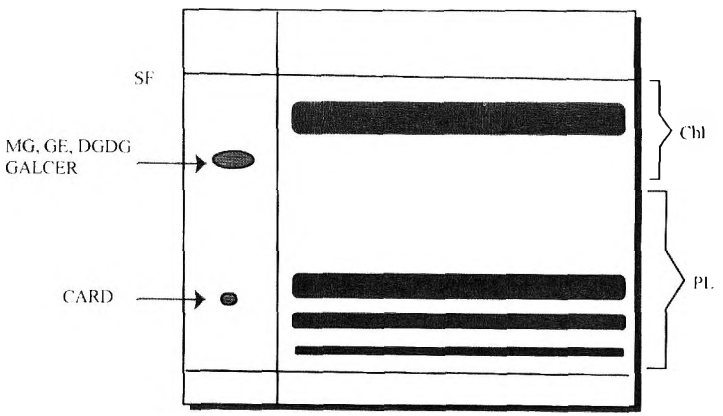


Figure 2. TLC development of standard lipids and of lipids from nettle in acetone/methanol/water (40:20:1).
SF: solvent front; MG: monoglycerides; GE: glycerylethers; DGDG: digalactosyldiglycerides; CARD: cardiolipin; GALCER: galactocerebrosides; Chl: chlorophylles (pigments); PL: polar lipids.

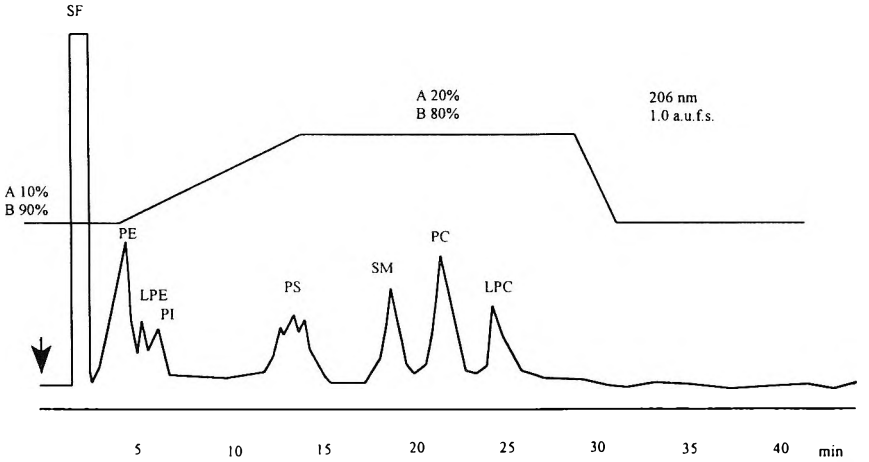


Figure 3. HPLC chromatogram of phospholipids standards on a HPLC silica B/5 column. Conditions and solvents in Results section. Gradient as indicated.
SF: solvent front; PE: phosphatidylethanolamine; LPE: lyso-phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine.

Chromatography

HPLC was performed on a dual pump Jasko (Tokyo, Japan) model 880-PU HPLC, supplied with a 330 μL loop Rheodyne (P/N 7125-047) injector. A Jasko model 875 UV spectrophotometer was used as detector (205 - 210nm). The spectrophotometer is connected to a Hewlett-Packard (Avondale, PA, USA) model HP-3396A integrator-plotter. The following columns were used: a cation exchange column SS 10μm Partisil 25cm x 4.6mm I.D., PXS 10/25 SCX from Whatman (Clifton, NJ, USA); an absorption column Silica 25cm x 4.6 mm I.D., H5 from Hichrom (Reading, Berkshire, U.K.); an absorption column Silica 25cm x 4.6mm B/5, Perkin- Elmer, (Norwalk, CT, USA); and a reverse phase column Nucleosil-300, C_{18} column 7μ, 250 x 4mm I.D. from Analysentechnik (Mainz, G). The flow rate was 1mL /min.

RESULTS

Extraction

Total lipids were extracted from the sample according to Bligh-Dyer[4]

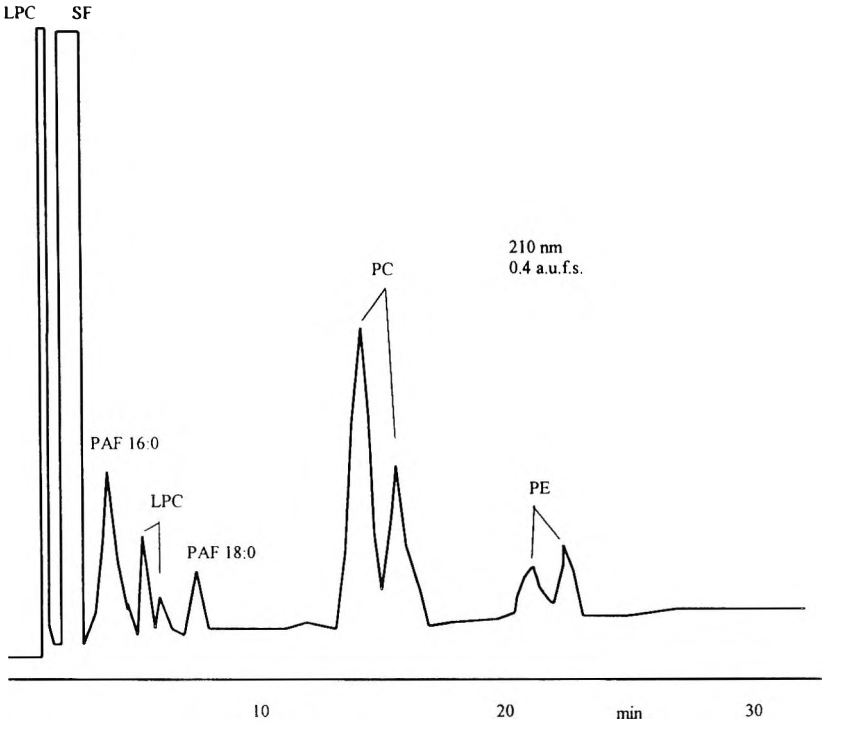


Figure 4. HPLC chromatogram of phospholipids standards on a HPLC nucleosil-300 column. Conditions and solvents in Results section.
SF: solvent front; PE: phosphatidylethanolamine; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine; PAF: Platelet-Activating Factor.

except that distilled water was used instead of saline. The chloroform phase from the extraction procedure was evaporated to dryness at 35 °C in a flash evaporator. The dry residue was transferred into a test tube using small volumes of chloroform/methanol (1:1), and the sample of crude total lipids was dried under a stream of nitrogen.

Isolation of Pigment-Free Total Lipids

In the case of samples of plant origin, the pigments were separated from total lipids with the aid of two successive TLC systems as follows: Crude

total lipids are redissolved in a small volume of chloroform/methanol (1:1) and applied to an appropriate number of preparative TLC plates. The chromatogram is developed in petroleum ether/benzene/glacial acetic acid (30:70:2). As shown in Figure 1 polar lipids (phospholipids and glycolipids) along with the pigments remain in the origin while neutral lipids mainly migrate along the plate with the exception of monoglycerides (MG) and glycerylethers (GE) which comigrate with glycolipids. The bands of neutral lipids as well as the band of polar lipids and pigments are scrapped off separately, extracted according to Bligh-Dyer, centrifuged and the organic solvents are phased by adding appropriate volumes of chloroform and water to arrive to a final chloroform/methanol/water ratio of 1: 1:0.9. The extract that contains polar lipids, small amount of neutral lipids and pigments is evaporated to dryness and redissolved in a small volume as above. Polar lipids and pigments are rechromatographed on an appropriate number of preparative TLC plates, using acetone/methanol/water (40:20:1) as developing system. As shown in Figure 2 pigments migrate near the solvent front while polar lipids migrate along the plate. Glycolipids as well as MG and GE migrate below pigments. The fraction of pigments is discarded while the polar lipids along with the small amount of neutral lipids are recovered as above. The fraction of polar lipids and neutral lipids are pooled together to reconstitute the total lipid extract free from pigments. The last step is necessary because glycolipids are partitioned in both fractions of neutral and phospholipids in the first developing system and polar lipids contain also of a small amount of neutral lipids (MG, GE) in the second development system.

Application of the described procedure was successfully performed to nettle leaves and roots (Figure 1, 2).

Separation of Total Lipids in Neutral, Glyco- and Phospholipids

An amount of 0.8g of pigment-free total lipids is redissolved in 0.5mL of chloroform/methanol (1:1), then taken up with up to 2mL chloroform and fractionated on a silicic acid column. Neutral lipids are eluted with 8 bed-volumes of chloroform followed by 2 bed-volumes of chloroform/acetone (19:1). Glycolipids are eluted with 2 bed-volumes of chloroform/acetone (2:1) followed by 17 bed-volume of acetone. Finally, phospholipids are eluted with 10 bed-volumes of methanol. The fractions of neutral, glyco- and phospholipids are evaporated to dryness and redissolved in a small volume of chloroform/methanol (1:1) as described above.

Application of the described procedure was successfully performed in the total lipids from bovine brain and from nettle leaves and roots.

Separation of Neutral Lipids into Classes and Species

Neutral lipids are separated into classes and species by HPLC[5] using a nucleosil-300, C_{18} column and a stepped gradient elution with the following solvents: A, methanol/water (80:20); B, acetonitrile/methanol (60:40); C, acetonitrile/tetrahydrofuran (99.5:0.5); and D, isopropanol/acetonitrile (99:1). A linear gradient from solvent A to solvent B in l0 min, a hold for 5 min in B and then a linear gradient to solvent C in l0min followed by a second hold in C for 15min results in a total separation within 40min (see Figure 6). By introducing a third linear gradient step from solvent C (decreasing the hold step to 5min) to solvent D in another l0min and holding D for 15min, a more distinct separation of triglycerides can be achieved. The flow rate is lmL/min and detection is achieved at 206nm with UV detector.

Separation of Glycolipids

Glycolipids are separated into classes and species by HPLC[6] using a nucleosil-300, C_{18} column and a stepped gradient elution, which starts with a linear gradient from 100% methanol/water (4:1) to 100% acetonitrile/methanol (7:5) in l0min and then hold for 15min. A 25min elution is sufficient for the separation of four glycolipid classes (gangliosides, sulfatides, digalactosyl-diglycerides and galactosyl-cerebrosides) which are studied. The flow rate is lmL/min and a UV detector is used at 206nm.

Separation of Phospholipids:

a) Into classes. Phospholipids are fractionated (Figure 3) on a Silica B/5 column with a stepped gradient elution with the following solvents: A, water; B, hexane/isopropanol (43:57). A hold for 5min in A 10%, a linear gradient from A 10% to A 20% in l0min, a hold for 15min in A 20% and then a linear gradient to A 10% in 2min and hold for l0min in A 10%. During this separation the flow rate is 2mL/min and phospholipids are detected at 206nm with UV detector. By this method phospholipids are satisfactorily separated within 25min.

b) Into species. Each class of phospholipids can be further fractionated into species with a nucleosil-300, C_{18} column and isocratic elution system of methanol/water/acetonitrile (63:7:30). The flow rate is lmL/min and the detection was achieved at 210nm with UV detector.

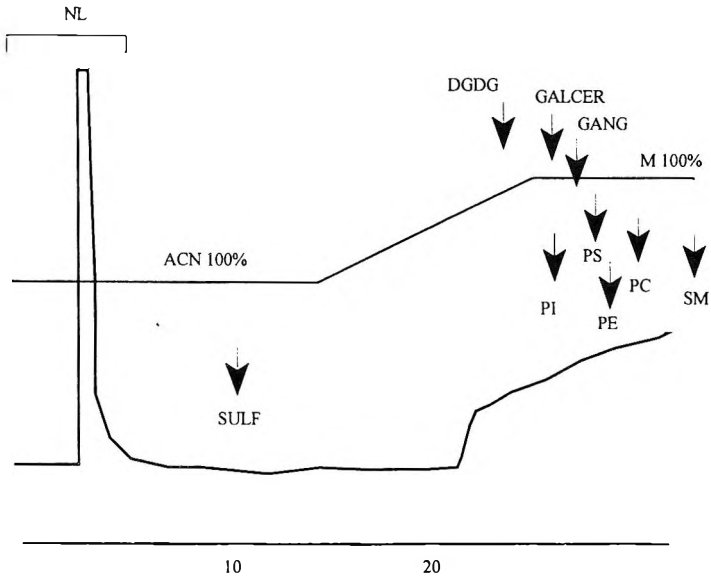


Figure 5. Separation of total lipids into neutral, glyco- and phospholipids on a HPLC silica column. Conditions and solvents in Discussion section. Gradient as indicated. NL: neutral lipids; SULF: sulfatides; DGDG: digalactosyldiglycerides; GALCER: galactocerebrosides; GANG: gangliosides; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin; PC: phosphatidylcholine.

The separations of phosphatidylethanolamine (PE), phospatidylcholine (PC), lysophosphatidylcholine (LPC) from human blood as well as the separation of semisynthetic PAF into species are shown in Figure 4. This method has the advantage of separating the more polar phospholipids (e.g. LPC, PAF) as well as the less polar ones (e.g. PE) into species within a reasonable time (25min).

DISCUSSION

The extraction of lipids can be performed by several conventional procedures. According to our experience, extraction according to Bligh-Dyer using distilled water instead of saline results in the recovery of gangliosides in the chloroform phase along with total lipids. Gangliosides can be separated from total lipids by washing the above chloroform phase with equal volume of aqueous phase (chloroform/methanol/saline, 3:48:47) where gangliosides are

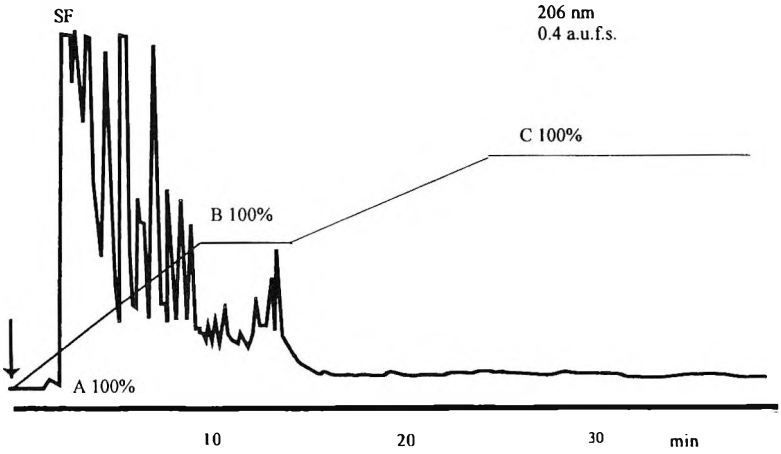


Figure 6. HPLC chromatogram of a neutral lipid fraction from nettle extracts on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front

partitioned in the water phase. By the addition of one volume of saline, one volume of ethanol and two volumes of chloroform in the water phase which contains gangliosides, in order to obtain a final ratio of chloroform/methanol/ethanol/saline (2:1:1:2), gangliosides, after two to four hours in 4 °C, are partitioned in the organic phase.[7] The last procedure has the advantage of obtaining gangliosides using mild techniques in an organic phase which is easily handled.

The existence of pigments in the sample which are usually isolated along with the total lipids is a problem that can be faced with the aid of the proposed new two-step TLC separation. By this way the fraction of pigments does not contain any lipids and can be discarded.

As already mentioned, neutral and glycolipids are present in both lipid fractions extracted from TLC. Therefore it was preferred to reconstitute the total lipid mixture free of pigments and then separate the pure neutral, glyco- and phospholipid fractions by the silicic acid column chromatographic technique. This technique is a useful procedure for fractionation of lipids mixtures in a preparative scale. The modified bulk elution system proposed here in comparison with the already published ones,[8] has the advantage of obtaining pure neutral, glyco- and phospholipid fractions with significantly

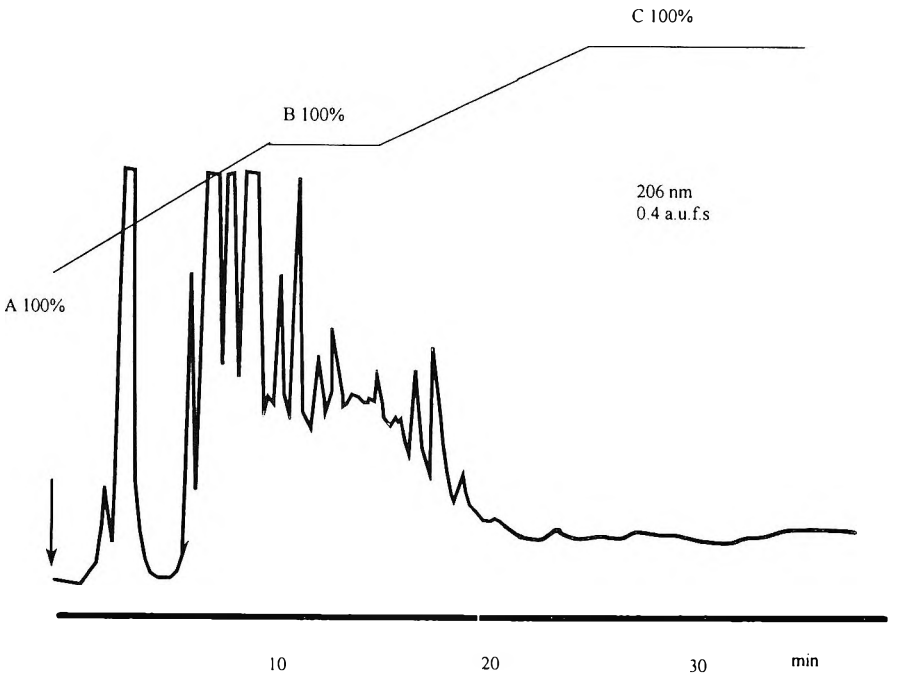


Figure 7. HPLC chromatogram of a neutral lipid fraction from human blood on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front.

reduced volumes of eluting solvents. Alternatively, when the sample contains small amount of lipids and at the same time our interest is focused on one of the lipid classes (either neutral, glyco- or phospholipids), or in the case we want to see a general pattern of total lipids (Figure 5), separation of total lipids can be performed on a HPLC absorption column, Silica from Hichrom H5 using a gradient elution system consisting of acetonitrile and methanol. A hold of acetonitrile 100% in 15min, followed by a linear gradient to methanol 100% in 10min and a last hold of methanol 100%. Neutral lipids are eluted along with the solvent front while phospholipids are eluted with 100% methanol. The elution of glycolipids begins approximately 3min after the elution of neutral lipids and is completed 3min after introduction of 100% methanol.

Several procedures for the separation of neutral lipids have previously been reported.[5] The method of the fractionation of neutral lipids proposed here,

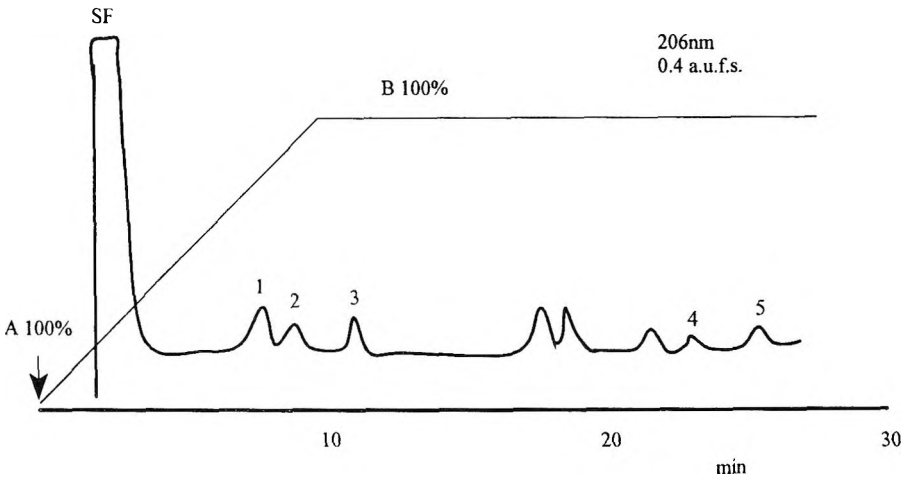


Figure 8. HPLC chromatogram of a glycolipid fraction from bovine brain on a HPLC nucleosil-300 column. Conditions and solvents as indicated in the Results section. SF: solvent front. 1: gangliosides; 2 and 3: sulfatides; 4 and 5: digalactosyl- diglycerides.

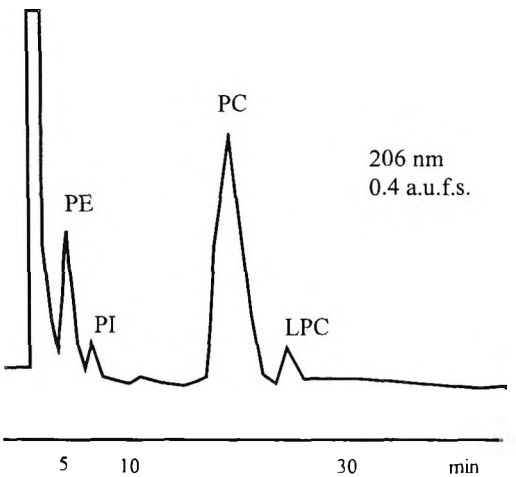


Figure 9. HPLC chromatogram of a phospholipid fraction from nettle extracts on a HPLC silica B/5 column. Conditions and solvents as indicated in the Results section. PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine; LPC: lyso-phosphatidylcholine.

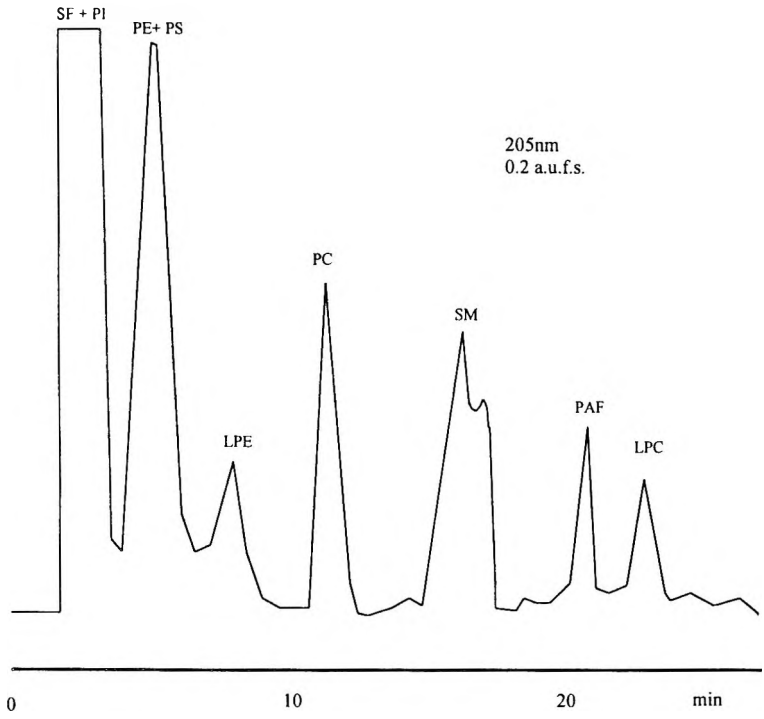


Figure 10. HPLC chromatogram of phospholipid standards on a HPLC cation exchange column. Conditions and solvents as indicated in the Discussion section.
SF: solvent front; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; LPE: lyso-phosphatidylethanolamine; PC: phosphatidylcholine; SM: sphingomyelin; PAF: Platelet-Activating Factor; LPC: lyso-phosphatidylcholine.

has the advantage of separating them simultaneously into classes and species, within the reasonable time of approximately lhr and with UV detection which has low cost, easy operation and is compatible with gradient elution. In addition, peak(s) of the same or different classes can be collected either for investigation of the structure and the possible biological activity or for further fractionation by conventional methods suitable for each class. Application of the described method was succesfully performed in neutral lipids derived from nettle (Figure 6) and in neutral lipids derived from human blood (Figure 7).

Glycolipids consist of molecules with a wide spectrum of polarity and for this reason the methods reported focus on the separation of individual

classes from different sources.[6] The recommended method is superior since it permits simultaneously the sufficient separation of glycolipids classes and species. Application of the described method in glycolipids derived from bovine brain is illustrated in Figure 8.

A number of methods concerning the separation of phospholipids into classes has been reported during the last decade.[9] Most of these methods permit a satisfactory separation of phospholipids. The only reason we recommend our method is that on one hand the reported methods do not have additional advantages compared to the proposed one and on the other hand this method has been well tested and used on a routine basis in our laboratory. Application of the described separation in phospholipids from nettle is presented in Figure 9. The most satisfactory separation of polar phospholipids such as sphigomyelin, PAF, lysophosphatidylcholine and lyso- PAF[10] can be accomplished by using a cation exchance column which permits an alternative method of phospholipid separation. In this method (Figure 10) an isocratic elution system is used consisting of acetonitrile/methanol/water (300:150:35).

Methods for the separation of an individual phospholipid class to its species have evolved rapidly in recent years.[9] In most cases a reverse phase HPLC is used with an elution system that consists of combinations of methanol, water, acetonitrile, acetic acid, chloroform etc. In the present method the achieved separation of the phospholipids into species is comparable to the previously reported ones.

In some cases, the separation of phosphonolipids from their structurally related phospholipids is necessary, in order to investigate phosphonolipids from different natural sources. This separation has been achieved by a HPLC method[11] which presents not only a satisfactory separation of two standard synthetic phosphonolipids from other structurally related phospholipids (PE, PC, SM, etc.) but also presents a method for the separation of phosphonolipids where phospholipids are not present.

REFERENCES

1. C. A. Demopoulos, R. N. Pinckard., D. J. Hanahan, J. Biol. Chem., **254**, 9355-9358 (1979).

2. C. A. Demopoulos, N. K. Andrikopoulos, S. Antonopoulou, Lipids, **29**, 305-309 (1994)

3. D. S. Galanos, V. M. Kapoulas, J. Lipid Res., **3**, 134-137 (1962).

4. E. G. Bligh, W. J. Dyer, Can. J. Biochem. Physiol., **37**, 911-917 (1959).

5. S. Antonopoulou, N. K. Adrikopoulos, C. A. Demopoulos, J. Liquid Chromatogr., **17**, 633-648 (1994).

6. C. A. Demopoulos, M. Kyrili, S. Antonopoulou, N. K. Andrikopoulos, J. Liquid Chromatogr., (submitted for publication, 1995).

7. S. E. Tsamperis, V. M. Kapoulas, C. A. Demopoulos, Hellen. Biochem. Biophys. Soc. Newsletter, **21**, 7-8 (1984).

8. G. Rouser, G. Kritchevsky, A. Yamamoto, "Column Chromatographic and Associated Procedures for Separation and Determination of Phosphatides and Glycolipids", in **Lipid Chromatographic Analysis 3**, G. V. Marinetti, ed., Marcel Dekker, Inc., New York, 1976, pp. 713-776.

9. W. C. Christie, "Methods for the Separation of Individual Phospholipid Classes", in **High performance Liquid Chromatography and Lipids**, Pergamon Press, 1987, pp. 106- 132.

10. N. K. Andrikopoulos, C. A. Demopoulos, A. Siafaka-Kapadai, J. Chromatogr., **363**, 412-417 (1986).

11. M. C. Moschidis, N. K. Andrikopoulos, J. Chromatogr., **403**, 363-365 (1987).

Received June 13, 1995
Accepted July 25, 1995
Manuscript 3905

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 537–545 (1996)

ANALYSIS OF ANTHOCYANINS BY CAPILLARY ZONE ELECTROPHORESIS

P. Bridle,[+] C. García-Viguera,* F.A. Tomás-Barberán

Lab. Fitoquímica
Dept. Ciencia y Tecnología de Alimentos
CEBAS-CSIC Apdo.Correos 4195
30080 Murcia, Spain.

ABSTRACT

A simple mixture of anthocyanins was analysed by CZE, using a standard silica capillary with a pH 8 borate running buffer. Generally, the anthocyanins separated according to the nature of the aglycone and degree of glycosylation. A concentrated injection solution was required for detection at 580 nm, due to the small proportion of coloured quinonoidal base in the anthocyanin equilibrium mixture at this pH, the small injection volume and the short detector path-length used in this technique.

It was observed that the ability to maintain a satisfactory current during analysis was critically dependent on the pH of the injection solution.

[+] Permanent address: Institute of Food Research, Reading Laboratory, Whiteknights Road, Reading RG6 6BZ, U.K.

INTRODUCTION

The CZE analysis of various groups of flavonoid compounds has been reported by several authors. SEITZ et al.[1] used this technique to separate pharmaceutical flavonols, McGHIE[2] analyzed sugarcane flavones and GIL et al[3] studied phenolic compounds in Spanish red wines. The effect of molecular structure on the electrophoretic mobility of flavonols was reported by McGHIE and MARKHAM.[4] The effect of different aglycone substituents and glycosylating groups on the CZE separation of flavonol-7-glycosides was described by MORIN et al.[5-6] A comparison of HPLC and CZE analysis of non-colored phenolics in red wines has recently been completed.[7] Other authors[8-12] have studied flavonoids, using micellar electrokinetic capillary chromatography (MECC) and isotacophoresis.[13] As far as we are aware, this is the first report describing the analysis of anthocyanins by CZE

Anthocyanin analysis using traditional chromatographic techniques is well established[14-15] and high performance liquid chromatography (HPLC) is now a standard technique for analyzing anthocyanins and other natural food colorants,[15-16] offering good separation and excellent quantitation. However, with the advent of capillary electrophoresis, a completely new technique for the separation of a wide variety of natural products has emerged. This preliminary study aimed to discover whether it was possible to analyze anthocyanins by CZE, as a prologue for a future comparison with other techniques.

MATERIALS AND METHODS

Capillary electrophoresis was performed using a Beckman P/ACE System 5510, with diode-array detection at 580 nm (detector path-length 75μm). A fused-silica capillary, 50 cm (to the detector) x 75 μm I.D., was used at 25°C, with a running buffer of 150 mM sodium borate (pH 8). Anthocyanins were introduced by hydrodynamic injection for 2 s (9nL injected) and run at a voltage of 25 kV, producing a current of approximately 45μA. The system was configured to run from anode to cathode. Electropherograms were processed using Beckman System Gold , PC-based chromatography data system software. All anthocyanin standard compounds used were purified from plant sources[17] in our laboratory at Reading, except malvidin 3,5-diglucoside (malvin) (Aldrich Chem. Co., Wisconsin, USA). Anthocyanins were dissolved in a mixture of 25 mM phosphate buffer (pH 2.5) and methanol (3:1). A suitable solute concentration for detection using our conditions was 1.2 mg/mL of each compound (2.5 mM expressed as malvidin 3-glucoside). The capillary was conditioned by washing with methanol for 5 min followed by freshly prepared 1

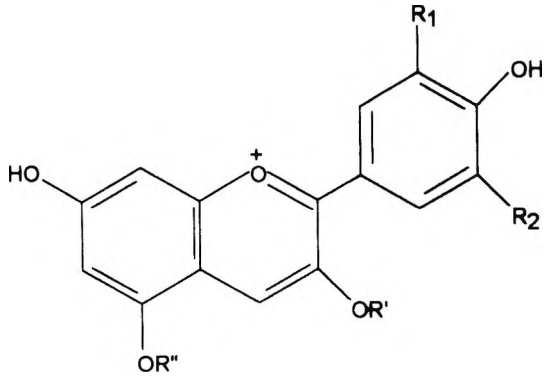
M sodium hydroxide (5 min), 0.1 M sodium hydroxide (5 min), distilled water (3 min) and fresh electrophoretic buffer (3 min). To optimize migration time and peak shape reproducibility, the capillary was flushed between analyses with 0.1 M sodium hydroxide (3 min) and distilled water (2 min).

RESULTS AND DISCUSSION

Anthocyanin standards were selected for analysis by CZE on the basis of their differing structural features (e.g. degree of hydroxylation or methoxylation and class of glycosidic substituent) so that any relationship between structure and electrophoretic behavior might be readily established. (Figure 1)

Analyses were performed at pH 8. Anthocyanins can exist in several equilibrium forms in aqueous solution, depending on the pH; the nature of these structures has been thoroughly discussed.[18-20] The red-colored flavylium cation is the familiar anthocyanin form which predominates at low pH values. Hence all chromatographic methods (paper (PPC), thin layer (TLC), column (CC) and HPLC) for anthocyanin analysis are based on acidified solvent systems, where a low pH is maintained. This colored anthocyanin species is particularly easy to detect selectively from other (colorless) compounds, either visually (PPC, TLC, CC) or at a visible wavelength setting on a HPLC detector. At higher pH values, the colorless carbinol base dominates in an equilibrium with the chalcone; near neutral pH and above, the blue-colored quinonoidal form also makes a small contribution to the total anthocyanin concentration. Thus, freshly made neutral or alkaline solutions are violet or blue, but their colors fade slowly. Furthermore, at pH values above neutrality, ionization of hydroxyl groups on the flavylium molecule may occur; depending on the position of hydroxyl substitution on the molecule, pKa values have reported to lie between 8.2 and 12.5 for analogous flavonols.[4] However, it should be noted that it is impractical to obtain accurate values for anthocyanins bearing multiple ionisable hydroxyl groups, since observation of deprotonation in alkaline solution is obscured by the instability of the pigment, which more or less rapidly disappears to a colorless equilibrium form.[21]

Various CZE parameters can be selected for analyzing anthocyanins, but in this first approach, we used analytical conditions similar to those used successfully for the analysis of non-colored phenolics in red wines.[3] Other variants in methodology are currently being explored, and we will report on these in due course. The separation of anthocyanins under these conditions is affected by three factors: charge-mass ratio, the influence of the electro-osmotic flow (EOF) and the possibility of borate (buffer) complexation, since borate is



R_1	R_2	R'	R"	Compound
H	H	Glucose	H	Pelargonidin 3-glucoside
OCH_3	OCH_3	Glucose	H	Malvidin 3-glucoside
OCH_3	OCH_3	Glucose	Glucose	Malvidin 3,5-diglucoside
OH	H	Glucose	H	Cyanidin 3-glucoside
OH	H	Rhamnose-glucose	H	Cyanidin 3-rutinoside
OH	OH	Glucose	H	Delphinidin 3-glucoside

Figure 1. Structures of anthocyanins.

known to complex readily with ortho-dihydroxyl groups.[22] It is evident from pH equilibria theory, that the anthocyanins should be present as neutral or negatively charged (ionization of hydroxyl groups) species, running from anode to cathode in the CZE capillary at varying rates according to charge density and influence of EOF. Under our conditions (CZE with borate buffer), anthocyanins with the highest negative charge density (charge/mass ratio) will have the greatest electrophoretic mobility (migration towards the anode), and therefore will be more strongly retained in the capillary. The main driving force under these conditions is the EOF, which causes all the anthocyanins in

the sample to elute at the cathode (detector end of the column). Anthocyanins with the least negative charge density elute first; conversely, anthocyanins having the greatest number of ionized phenolic hydroxyls at the buffer pH, and those which are able to complex with borate, will have a greater negative charge and be more strongly retained on the capillary, consequently eluting with longer migration times.

The effect of increasing glycosylation in anthocyanins with the same aglycone, is to shorten the migration time (MT), since the charge/mass ratio is lowest in the most highly glycosylated forms (Figure 2). This is illustrated by comparing malvidin 3,5-diglucoside (MT=5.07) with malvidin 3-glucoside (MT=6.34) or cyanidin 3-rutinoside (MT=6.79) with cyanidin 3-glucoside (MT=7.26); the anthocyanins with the greater mass (but the same charge), elute with shorter migration times in both examples. These results accord well with earlier studies of CZE analysis of flavonol O-glycosides.[23-24]

When examining the migration order of the four anthocyanin 3-monoglucosides, the behaviour of malvidin 3-glucoside (MT= 6.34) compared to pelargonidin 3-glucoside (MT= 6.30), is not explained by the charge/mass ratio theory. In this case, both anthocyanins have the same number of ionizable phenolic hydroxyls and the same possibilities for borate-complex formation; however, malvidin 3-glucoside has the lower charge/mass ratio due to the higher molecular weight of malvidin compared with pelargonidin. Thus, theoretically, malvidin 3-glucoside should elute with a shorter MT than pelargonidin 3-glucoside. However, the reverse situation occurred (Figure 2). Thus, another explanation is required for this anomalous behavior; for instance, it is possible to speculate that the acidity of the 4'-hydroxyl of both anthocyanins may differ due to the presence of adjacent methyl ethers in malvidin 3-glucoside. This could affect the ionization of this hydroxyl group and hence the overall electrophoretic mobility.

The migration order of the remaining 3-monoglucosides accords with theory; consequently the increase in migration time of cyanidin 3-glucoside (MT=7.26) and delphinidin 3-glucoside (MT=7.41), when compared with pelargonidin 3-glucoside (MT= 6.30), is due to the additional free phenolic hydroxyls on the B-ring, capable of ionization at pH 8. Additionally, the possibilities of borate-complex formation are increased, thus delphinidin 3-glucoside elutes later than cyanidin 3-glucoside, which in turn elutes later than pelargonidin 3-glucoside.

The pH of the solvent used for sample preparation is of vital importance. To dissolve the anthocyanins for analysis, it was necessary to use a low pH

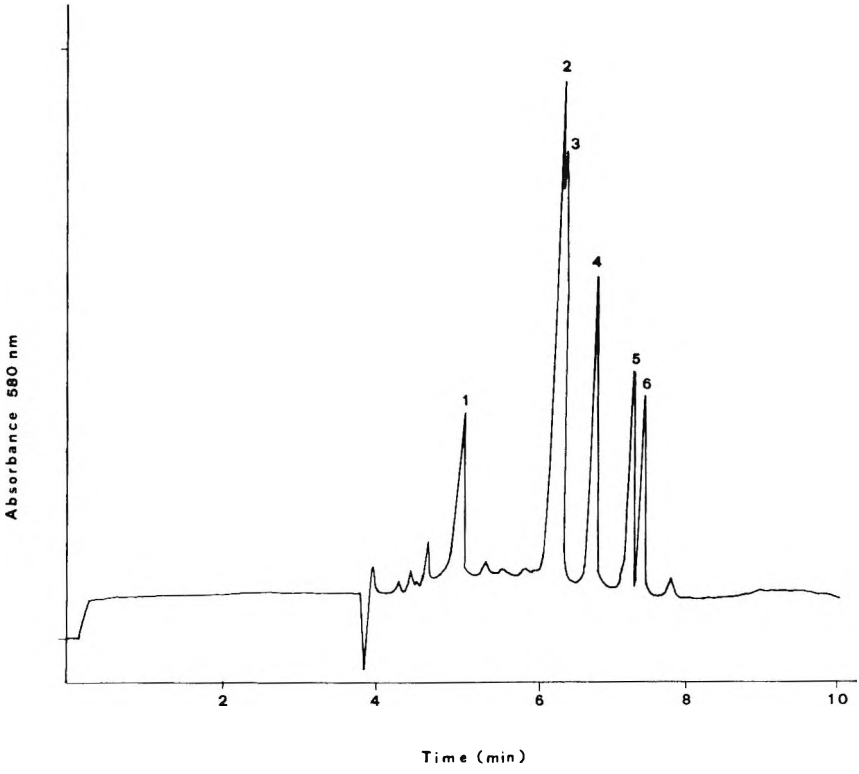


Figure 2. CZE electropherogram of anthocyanin standards. 1) Malvidin 3,5-diglucoside, 2) Pelargonidin 3-glucoside, 3) Malvidin 3-glucoside, 4) Cyanidin 3-rutinoside, 5) Cyanidin 3-glucoside, 6) Delphinidin 3-glucoside.

buffer (pH 2.5), with or without an addition (<25%) of methanol. Our general observation was that 3-glycosides dissolved more readily than 3,5-diglucosides. These conditions provided a good compromise between dissolving the anthocyanin at a pH so high that the sample became unstable and degraded on standing, and a pH so low that there was insufficient running current after injection. Even traces of (formic) acid in the injection solvent had a deleterious effect on the current.

Peaks were adequately monitored at 580nm, a suitable wavelength for detecting the quinonoidal anthocyanin form. The exact pH of the running sample is not certain, but we believe it was very close to pH 8, since the CZE spectrum of cyanidin 3-glucoside matched closely that of the anthocyanin

dissolved in pH 8 buffer and measured on a spectrophotometer. Similar CZE spectra were recorded for the other anthocyanins analyzed. It is difficult at this stage, to estimate how useful spectra taken at pH 8 could be for characterizing different anthocyanins, compared to the more familiar and distinctive spectra typically obtained at acidic pH values. However, in this first approach, the main objective was to obtain a separation of the different anthocyanins, and this means of detection gave adequate information to achieve this end.

In conclusion, we were able to obtain satisfactory separation of a simple anthocyanin mixture at pH 8 using CZE with spectrophotometric detection. The optimization of the analytical parameters for the separation of this interesting group of compounds in an acidic buffer is currently being investigated.

ACKNOWLEDGEMENTS

The authors wish to thank the Spanish Ministerio de Educación y Ciencia and the British Council for financial support (Acciones Integradas 1994/6: 155 B-220B & 561-1338), also J. Aliphon (UK), A. Díez-Cascón and E. Torija (Spain), Beckman Instruments, for technical advice and discussion.

REFERENCES

1. U. Seitz, P.J. Oefner, S. Nathakarnkitkool, M. Popp, G.K. Bonn, Electrophoresis, **13**, 35-38 (1992).

2. T.K. McGhie, J. Chromatogr., **634**, 701-772 (1993).

3. M.I. Gil, C. García-Viguera, P. Bridle, F.A. Tomás-Barberán, Z. Lebensm.Unters Forsch., **200**, 278-281 (1995).

4. T.K. McGhie, K.R. Markham, Phytochem. Anal., **5**, 121-126 (1994).

5. Ph. Morin, F. Villard, M. Dreux, J. Chomatogr., **628**, 153-160 (1993).

6. Ph. Morin, F. Villard, M. Dreux, P. Andre, J. Chromatogr., **628**, 161-169 (1993).

7. C. García-Viguera, P. Bridle, Food Chem., **54** (in press) (1995).

8. P.G. Pietta, P.L. Mauri, A. Rava, G. Sabbatini, J. Chromatogr., **549**, 367-373 (1991).

9. C. Bjergegaard, S. Michaelsen, H. Sorensen, J. Chromatogr., **608,** 403-411 (1992).

10. C. Bjergegaard, S. Michaelsen, K. Mortensen, H. Sorensen, J. Chromatogr., **652**, 477-485 (1993).

11. C.L. Ng, C.P. Ong, H.K. Lee, S.F.Y. Li, Chromatographia, **34**, 166-172 (1992).

12. F. Ferreres, M.A. Blázquez, M.I. Gil, F.A. Tomás-Barberán, J. Chromatogr., **669**, 268-274 (1994).

13. U. Seitz, G. Bonn, P. Oefner, M. Popp, J. Chromatogr., **559**, 499-504 (1991).

14. G. Hrazdina, "Anthocyanins", in **The Flavonoids: Advances in Research**, Eds. J.B. Harborne, T.J. Mabry, Chapman and Hall, London and New York, 1982, pp.135-188.

15. D. Strack, V. Wray, "Anthocyanins", in **Methods in Plant Biochemistry, Vol. 1, Plant Phenolics**, Ed. J.B. Harborne, Academic Press, London, 1989, pp. 325-356.

16. A.G.H. Lea, "HPLC of Natural Pigments in Foodstuffs", in **HPLC in Foodstuff Analysis**, Ed. R. Macrae, Academic Press, London, 1988, pp. 277-337.

17. J.B. Harborne, "The Anthocyanin Pigments", in **Comparative Biochemistry of the Flavonoids**, Academic Press, London, 1967, pp 1-36.

18. R. Brouillard, J.E. Dubois, J. Am. Chem. Soc., **99**, 1359-1364 (1977).

19. R. Brouillard, B. Delaporte, J. Am. Chem. Soc., **99**, 8461-8468 (1977).

20. R. Brouillard, B. Delaporte, **Protons and Ions Involved in Fast Dynamic Phenomena**, Ed. P. Laszlo, Elsevier, Amsterdam, 1978, pp. 403-412.

21. R. Brouillard, " Chemical Structure of Anthocyanins" in **Anthocyanins as Food Colours**, Ed. P. Markakis, Academic Press, London, 1982, pp. 1-40.

22. K.R. Markham, "Ultraviolet-visible Absorption Spectroscopy" in **Techniques of Flavonoid Identification**, Academic Press, London, 1982, pp. 36-49.

23. Ph. Morin, M. Dreux, J. Liquid Chromatogr., **16,** 3735-3755 (1993).

24. F.A. Tomás-Barberán, Phytochem. Anal., **6**, 177-192 (1995).

Received April 27, 1995
Accepted September 15, 1995
Manuscript 3863

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 547–564 (1996)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSES OF SULPHONAMIDES AND DIHYDROFOLATE REDUCTASE INHIBITORS. II. SEPARATIONS IN ACETONITRILE MODIFIED SOLUTIONS, TERNARY GRADIENT STUDIES & FLOW PROGRAMMING

Maria C. Ricci,[#] Reginald F. Cross*

School of Chemical Sciences
Swinburne University of Technology
John Street, Hawthorn
Victoria 3122, Australia

ABSTRACT

Acetonitrile has been investigated as the organic modifier for the reverse phase separation of twenty-two sulphonamides and three commonly used dihydrofolate reductase inhibitors. Isocratic analyses indicate that 10% acetonitrile is approximately isoeluotropic with 16% methanol. This allows a comparison of selectivities. Relative to methanol, in gradient elution involving acetonitrile only, unfavourable changes in the relative retentions of some pairs of analyses outweighed the advantages of favourable selectivities. Insertion of acetonitrile into methanol gradients did not produce the improved separations expected. However, the imposition of a program of reduced flow upon an

[#]Current address: Perkin-Elmer Corporation, Applied Biosystems Division 1270 Ferntree Gully Road, Scoresby, Victoria 3179. Australia.

established methanol gradient demonstrated significantly increased efficiencies in the front half of the chromatogram. This permitted the resolution of the first thirteen compounds, including the difficult-to-separate pair of sulphathiazole and sulphapyridine.

INTRODUCTION

In a previous paper,[1] we presented the results of an investigation into the reverse phase retention behaviour of twenty-two sulphonamides (SFA) and the three commonly used dihydrofolate reductase inhibitors (DHFR) in methanol modified mobile phases. The effects of variation in the percentage of methanol and the pH were determined isocratically. Gradients were developed and then modified by variation of the concentration of the phosphate buffer. Significant variations in retention behaviour were observed such that the majority of combinations of drugs could be screened. However, no set of conditions studied gave rise to a total separation.

Methanol (MEOH) was chosen as the first organic modifier to examine, as there were no reports of detailed studies of its effects on the broad screening for SFA. Thus, any possible advantages in selectivity were unknown. Furthermore, the weaker reverse phase solvent (compared to acetonitrile (ACN)) permitted easier experimental fine tuning of the net solvent strength. However, whilst the MEOH work revealed sets of conditions conducive to most separations, the ultimate aim of the separation of all analytes was not achieved. Thus, we returned to ACN. ACN has been investigated as an organic modifier in the separation of the SFA before,[2,3] albeit not in conjunction with the particular stationary phase utilised in this study.

Given appropriate selectivity differences, there were two possible useful outcomes. The first was an outright separation of all of the drugs in ACN. The second possibilty was to incorporate ACN into a MEOH gradient if sufficient difference was evident between the solvents in the critical part(s) of the separation. In all attempts to separate a large number of the SFA, the major stumbling block has been those SFA eluted at moderate solvent strengths in the middle of the chromatogram. As this includes previous ACN studies (on other stationary phases), it was prudent to consider an alternate strategy.

Adjustment of the flow rate is generally regarded as an ancilliary tool. In its simplest application the last one or two strongly retained analytes may be more rapidly eluted.[4] This is particularly useful in isocratic analyses since

pressure (and thus flow) re-equilibration is rapid. None of the column characteristics, nor the nature of the variation of the plot of the height equivalent to a theoretical plate (H) versus flow rate,[5] nor the initial position on the curve are important.

Loss of efficiency is irrelevant if the analysis time is significantly reduced. At the front end of the chromatogram, additional separation time is provided by reducing the flow rate. Whilst the same increase in separation space may be achieved by an appropriate reduction in the solvent strength, we have experienced the combination of analyses and stationary phase for which the former provided a separation but the latter did not.[6] In this case the initial position on the H/flow rate curve does matter.

Excessive peak broadening due to the ascent of the steep section of the H versus flow rate plot at very low flow rates could be counter productive. Fortunately, the commonly adopted flow rates are often above the optimum so that we are protected from that eventuality. Flow rate programming has also been used for some time as the solution to the general elution problem in the analysis of compounds which are only conveniently detected by refractive index.[7] The general coupling of solvent and flow programming has also been systematically examined.[8]

EXPERIMENTAL

With the exception of the HPLC grade ACN, all chemicals, equipment and experimental methods were as previously described.[1]

The pH range of 2.5-3, found to be optimal by Roos,[2] and confirmed in Part I of this study, was used without further investigation. Similarly, the phosphate buffer found to yield the best separations in Part I of this study (0.001 M), was also utilised without further study.

Although not reported in Part 1, substitution of phosphate by acetate (in methanolic solutions) yielded poorer separations. The 0.001M KH_2PO_4 buffer solutions were adjusted to the pH range of 2.75-2.77 and the column oven was set to 30°C for all runs unless otherwise indicated.

The full names of the compounds are given in the first part of this study[1] and the structures of all of the drugs have also been listed previously.[9]

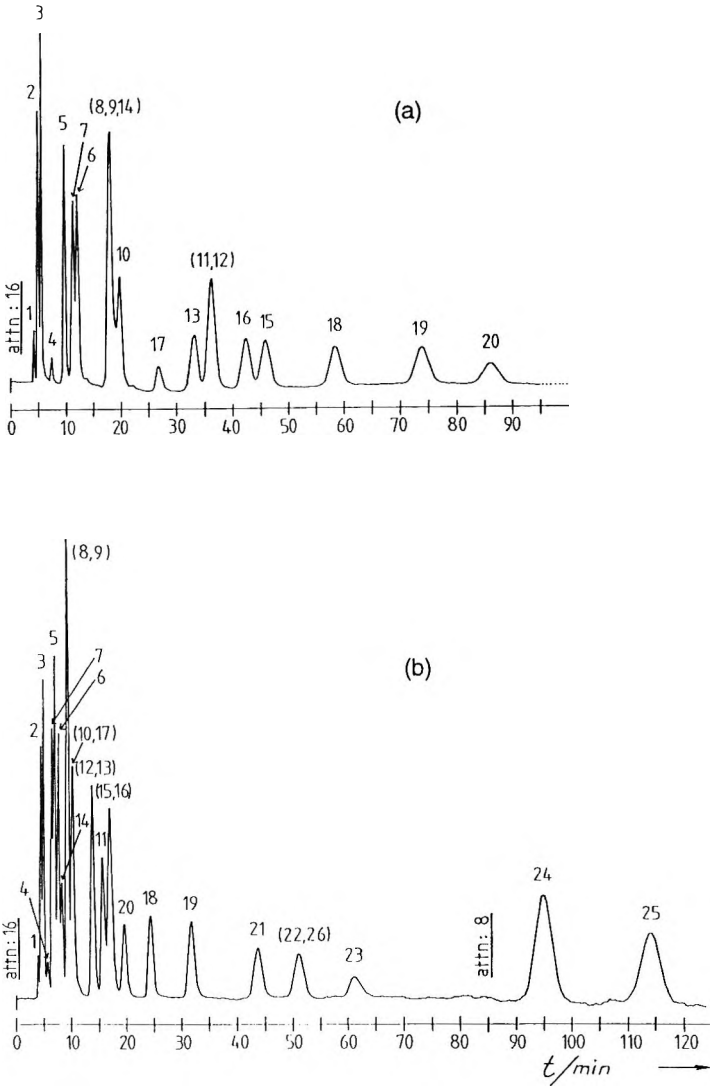


Figure 1. Isocratic acetonitrile (ACN)/0.001 M phosphate buffer (pH 2.75) chromatograms. (a) 5% ACN (thefirst 90 minutes). (b) 10% ACN.
The compounds are: (1)SNAC, (2)SG, (3)SAN, (4)SAM hydrolysis product, (5)SAC, (6)SDZ, (7)SISM, (8)ST, (9)SP, (10)SMRZ, (11)SM, (12)SAM, (13)SMAZ, (14)DVD, (15)SMIZ, (16)SMP, (17)TMP, (18)SCP, (19)SMOX, (20)SST, (21)SISX, (22)SB, (23)PST, (24)SDIM, (25)SQ, (26)PYR.

Table 1

Comparison of the Isocratic Elution Characteristics for the 22 SFA and 3 DHFR in 16% Methanol and 10% Acetonitrile

Time (Minutes)	16% Methanol (0.1M Phosphate Buffer, pH 2.8)	10% Acetonitrile (0.001M Phosphate Buffer, pH 2.8)
4.5	SNAC(1)	SNAC(1)
5	SG(2)&SAN(3)&SAM(HP)(4)	SG(2)
		SAN(3)
		SAM(HP)(4)
6.5		**SISM(7)**
7	SAC(5)	SAC(5)
8	SDZ(6)&**SISM(7)**	SDZ(6)
9	ST(8)	[DVD(14)]
	SP(9)	
10	SMRZ(10)	ST(8)&SP(9)
10.5		SMRZ(10)&.... [TMP(17)]
14	[DVD(14)&..**SM(11)**SAM(12)&SMAZ(13)	
14.5	SMIZ(15)	SAM(12)&SMAZ(13)
15	[TMP(17)]	
15.5		**SM(11)**
17	SMP(16)	
17.5		SMIZ(15)&SMP(16)
20	SCP(18)	**SST(20)**
21	**SST(20)**	
22	SMOX(19)	
25		SCP(18)
31	SISX(21)	SMOX(19)
35	SB(22)	
44		SISX(21)
51		SB(22)&..... [PYR(26)]
71, 61	PST(23)	PST(23)
82, 95	SDIM(24)	SDIM(24)
105, 115	SQ(25)	SQ(25)
123	[PYR(26)]	

RESULTS AND DISCUSSION

Analyses in Acetonitrile Modified Mobile Phases

(a) Isocratic

Three trial chromatograms were run. The ACN content of the mobile phase, the number of discernable peaks and the total analysis times were 5%: 22 peaks in 430 minutes, 10%: 21 peaks in 120 minutes and 15%: 16 peaks in 40 minutes, respectively. Figure 1(a) shows the chromatogram for the first 90 minutes of the 5% ACN run and Figure 1(b) shows the 10% run. The elution

characteristics for the 10% ACN isocratic run are compared with those for 16% MEOH[1] in Table 1. Column 1 is an approximate time scale with the later double times being those in 16% MEOH and 10% ACN, respectively. Columns 2 and 3 list the peaks in order of elution in 16% MEOH and 10% ACN, respectively. Individual compounds are listed where they are clearly discernable from neighbouring peaks. Otherwise, coeluting compounds are listed together, joined by an ampersand (&). In each list (columns 2 and 3) the DHFR have been put to the side since the observed differences in their elution characteristics have been demonstrated (near the conclusion of the first part of this study[1]) to be due to the decreased phosphate concentrations. Whilst there were changes in the relative retentions of several pairs of SFA when the phosphate buffer concentration was decreased from 0.1M to 0.001M, there are no changes in elution order amongst them.

A comparison of the retention data for the 16% MEOH and 10% ACN ioscratic chromatograms indicates these mobile phases to be approximately isoeluotropic. For the 16% MeOH run, a total of 20 peaks were separated in 125 minutes with a k' range of 0.5 - 43. Similarly, the 10% ACN run yielded 21 peaks in 114 minutes with a k' range of 0.4 - 41. Inspection of Table I reveals that the order of elution of the SFA is largely the same in both of the organic modifiers. Of the 22 SFA and the SAM hydrolysis product observable in these runs, 20 were clearly eluted in the same order. In ACN, SISM and SM are eluted earlier and later in that order, respectively, (and are underlined and in bold typeface in the table). SCP and SST are inverted in the elution orders (and are each singly highlighted in the table). Also, closer inspection of Table I reveals several changes in the relative retentions in ACN.

It should be noted from Table 1, however, that with approximately the same number of discernable peaks in each solvent system, the advantages of elution in 10% ACN (compared to 16% MEOH) were largely negated in other parts of the separation. Overall, the most positive change occurred around the 14 minute region of the chromatogram, from which DVD is removed, by the decreased salt concentration, and SM is removed by the differing selectivity of ACN. Since this had been the most difficult region of the separation, it was decided to investigate straight ACN separations.

Isocratic analyses were performed at 8-14% ACN, inclusive, and combined with the earlier data at 5, 10 and 15%. Figure 2 is the plot of log k' versus percentage ACN. At the high percentage ACN end, the plot is compressed and multiple coelutions occur. Working back from 15% ACN towards the less congested regions of the plot, there is another unfortunate feature of the data that becomes apparent. This is the large and non-systematic

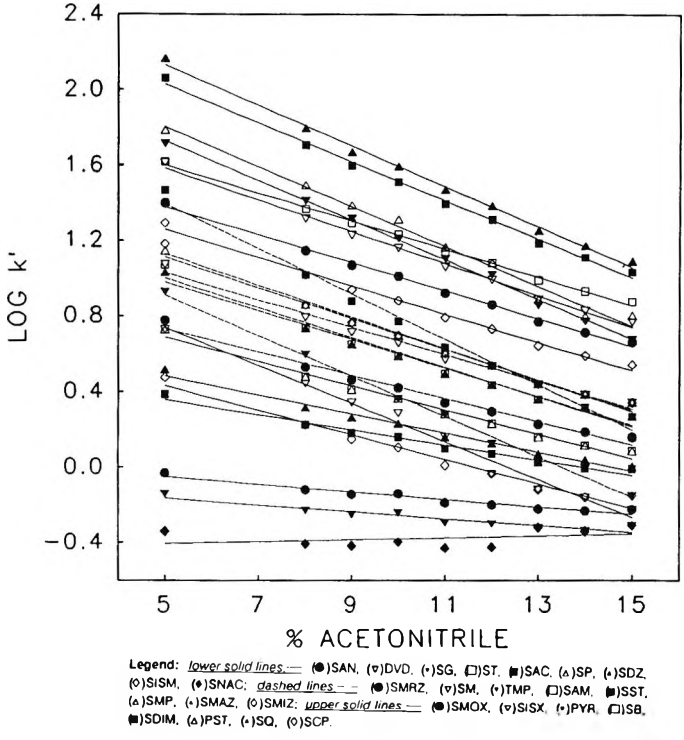


Figure 2. Plot of log k' vs % acetonitrile for the 22 SFA and 3 DHFR compounds using a 0.001M phosphate buffer at pH 2.75.

variation in d(log k')/d(% ACN) for many of the compounds. In the lower section of Figure 2, the gradient of the SISM plot is significantly greater than would be expected in that part of the figure. Slightly higher up in the figure, in the middle region, DVD, TMP and SST have gradients that are even greater, relative to the average at that k'. And, in the band of plots from log k' ≈ 1.6 at 5% ACN down to log k' ≈ 1 at 15% ACN, the gradients for the four compounds (SISX, SB, PYR and PST) vary around the values that would be anticipated in that part of the figure. This is a feature of the ACN analyses that is quite different from the MEOH data where far fewer compounds varied greatly from the overall trend in gradients. The effect of these variations is to cause multiple crossovers in the plots and a large number of coelutions between continually varying pairs of compounds.

From the severely congested end of Figure 1, around 15% and down as far as (and including) 8%, the result is that isocratic analysis looks very

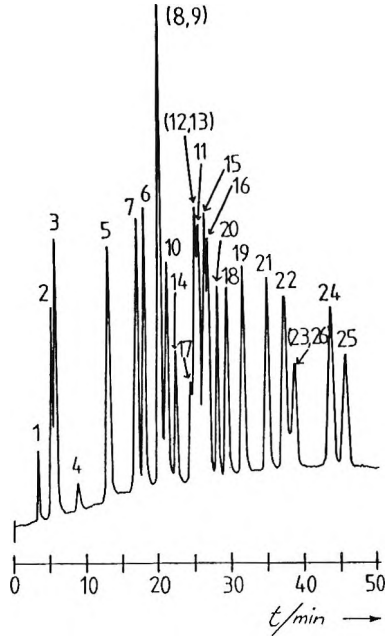


Figure 3. The best gradient chromatogram obtained with ACN/0.001M phosphate buffer (pH 2.75). The percentage of ACN varied with time as: 0 minutes, 0%; 5 min, 5%; 20 min, 15%; 35 min, 18%; 45 min, 30%; 55 min, 60%. The compounds are numbered as in Figure 1.

unattractive. At 7% ACN there is a narrow window of modifier concentration free of crossovers and, therefore, potentially useful. However, there are two persistent pairs of coeluting compounds (SAM(12) & SMAZ(13) and SMIZ(15) & SMP(16)) and the presumption of no other coelutions depends upon the accuracy of the software-drawn lines of best fit that appear not to be in good agreement with much of the 5% retention data. Furthermore, interpolation of the SQ plot to 7% ACN yields a total analysis time of 238 minutes, so no further investigations of isocratic ACN separations were attempted.

(b) Gradients

One of the early gradients to show promise was a simple linear variation from 0-15% ACN over 29 minutes, followed by isocratic elution. Compared to

Table 2

Gradient Program used for the Chromatogram Shown in Figure 4(b)

Time (Minutes)	%0.001M Phosphate	%MEOH	%ACN
0	100	0	0
0.1	95	5	0
7	92	8	0
7.01	94	0	6
21	94	0	6
26	84	16	0
31	82	18	0
36	70	30	0

the 10% isocratic separation, there was not any increase in the number of compounds resolved - and amongst the unresolved compounds - two of the most refractory coelutions remained (ST(8) & SP(9) and SAM(12) & SMAZ(13)). However, the total elution time for the 21 peaks was reduced from 120 minutes to 67.

After a large number of variations, the best gradient involved steps from 0-5-15-18-30-60 % ACN at 0, 5, 20, 35, 45 and 55 minutes, respectively. Figure 3 is the chromatogram obtained. With a further reduction of 20 minutes, two additional peaks were obtained. However, the cost of the faster separation was reduced resolution between some pairs. As this separation was still clearly inferior to the best acieved in MEOH, ACN was abandoned as a single modifier. The very high mobility with respect to % ACN of several of the analyses relative to the majority makes the estimation of the net effect of several gradient steps extremely difficult.

(c) Insertion in a methanol gradient

Towards the section (a), it was noted that an inconsistency existed between the extrapolated lines of best fit to the 15-8% ACN isocratic runs and the observed data at 5% ACN for several of the analytes, thus suggesting a possible systematic error in the latter. Aiming specifically at the difficult middle group of compounds (11-16), a 6% isocratic run was performed. Figure 4(a) is the chromatogram for the first 50 minutes and the middle group are all

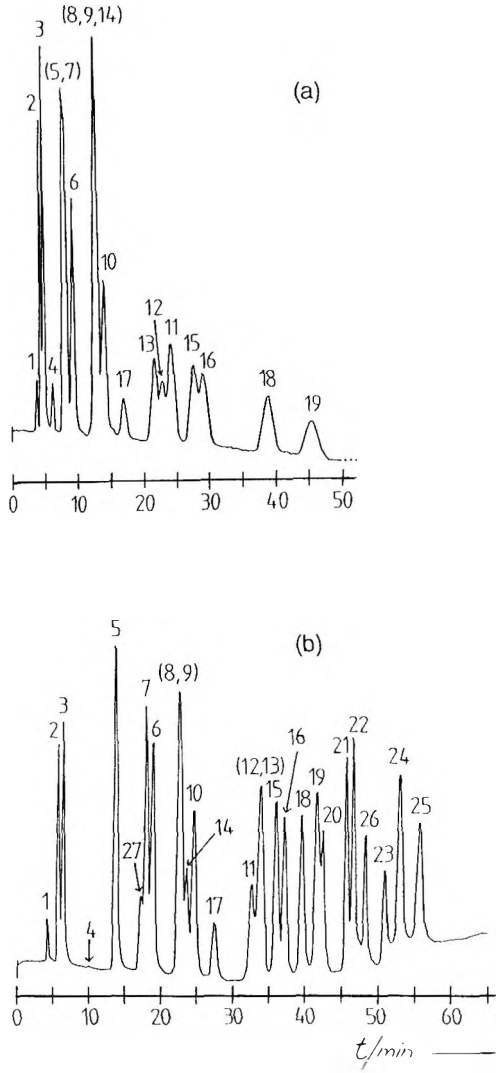


Figure 4. Chromatograms showing (a) isocratic 6% ACN elution and (b) the effect of insertion of 6% ACN in a MEOH gradient. The gradient is given in Table 2. The compounds are numbered as in Fiqure 1. 27 is the second SAM hydrolysis product.

partially resolved. The objective was to maintain the basic features of the best MEOH gradient but to replace MEOH with 6% ACN to elute compounds

11-16. Eventually, the gradient in Table 2 was settled upon. Figure 4(b) is the chromatogram. With a delay time of 6.57 minutes, the introduction of the 6% ACN at 7 minutes brings the ACN to the column head at 13½ minutes. The effect of this solvent switch is clearly evident around 19 minutes, where the reversal of compounds 6 and 7 is observed, in agreement with Table 1. This implies that the methanol gradient in the first 7 minutes was ineffectual in moving compounds 6 and 7 along the column. This might be expected to apply to the subsequent elutions as well. However, this is far from the case.

Comparison of Figures 4(a) and (b) indicates that MEOH causes the partial resolution of compound 14 from 8 & 9 and the improved separation of 10 from this group. Furthermore, compound 11 elutes before 12 - as is the case in methanolic solutions - rather than after compound 13 (as in ACN; see Table 1). Unfortunately, the difficult-to-separate-pair of compounds, 12 and 13, also reflect the influence of the MEOH and are again coeluted.

Increasing the Efficiency of the Separation

From the resolution equation,

$$R = 1/4\sqrt{N} \bullet \frac{\alpha - 1}{\alpha} \bullet \frac{k'}{k'+1}$$

it is clear that resolution may be improved by increasing any of N, α or k'. To this point in the investigation, we have largely been concerned with α and k'. The studies of the effect of percentage organic modifier are mainly an examination of variation in k' (with some changes in α), whereas the study of the effect of pH[1] is primarily looking at variations in selectivity (α) (with k' effects also). The change of organic modifier (MEOH to ACN) over the same acceptable range of k' values is purely an investigation of α.

Another option is to increase N. The most obvious way to do this is to increase the length of the column. In a previous study,[3] on a different stationary phase, columns of twice the initial length were prepared and the theoretical increase of 1.4 in the resolution was observed for the SFA. This increase in resolution is highly significant and would clearly be sufficient to resolve any refractory pair of compounds, once they were made discernable in the chromatogram as a two-compound peak, under any set of conditions.

However, the cost is in the doubled analysis time, which is generally unacceptable. A possible compromise involves using an increased flow rate through the longer column.

For a typical monomeric C_{18} bonded phase on microporous 5 μm silica, a doubling of the flow rate, from the vicinity of the optimum value, would only cause approximately a 15% decrease in efficiency.[10] Thus the net effect of doubling both the column length and the flow rate would be to totally offset the increased analysis time and retain a 25% increase in resolution. However, with the 60 cm packed capillary columns prepared for the earlier study,[3] excessive back pressures on the pump prevented this option. Similarly, the 60 cm packed capillary columns prepared for this study again caused excessive back pressures on the pump and could not be utilised in the desired fashion, especially with the more viscous methanolic solutions. As we wished to investigate increasing N whilst retaining the same stationary phase packing, adjustment of the flow rate remained the only available option.

Flow Programming

For the packed capillary columns used in this study (0.35 mm id), it is difficult to predict the effect of change in the flow rate from the literature. Much of the published data is based upon pioneering research which ranged over a large number of variables. The data of Dong and Grant[10] clearly demonstrated the progressive effect of reduction of particle size (10-5-3 μm) in conventional HPLC, and the relative shapes of the van Deemter plots provide the long accepted basis for fast LC. Similar data has been presented by Verzele and Dewaele,[11] for C_{18} phases bonded to 8, 5, 3 and 2 μm silica. However, at a fixed particle size (5 μm in this case) the extrapolation of height equivalent to a theoretical plate (H) versus flow rate (v) from conventional to capillary packed columns is problematic. Firstly, there is the difference between the reference data. In each case H_{min} is approximately 12 μm and approaches the theoretical limit of 2dp for efficiency, thus indicating that both columns are well packed[12] (dp = the diameter of the packing material.). However, the optimum values of the flow rate (v_{opt}) are significantly different; 1.2[10] and 0.7[11] mL min^{-1}, indicating, in this case, differences in mass transfer characteristics and the quality of the base silica.[12]

Secondly, there is the problem of extrapolation. The expected gains in efficiency due to more homogeneous packing and more uniform heat dissipation in packed capillaries[13] may also shift v_{opt}. However, Kucera's

excellent data[14] for 10 μm silica shows identical v_{opt} in 4.6 and 1.0 mm i.d. columns. Kucera's data indicates that, all other things being equal, v_{opt} should be the same for our packed capillary column as for the conventional columns containing 5 μm particles: Dong and Grant[10] 1.2 mL min^{-1}, or Verzele and Dewaele[11] 0.7 mL min^{-1}, in each case for 4.6 mm i.d. columns. Scaling this flow rate down to the equivalent for our 0.35 mm i.d. column predicts $v_{opt} \approx 7$[10] or 4[11] μL min^{-1}. As the measured flow rate was around 6 μL min^{-1}, with $v_{opt} \approx$ 7 μL min^{-1}, any significant change in the flow rate would be expected to give rise to a decrease in N and loss of resolution. On the other hand, with $v_{opt} \approx 4$ μL min^{-1} the flow rate could be reduced by >50% and efficiency gained. It is clear that, without detailed knowledge of the silica substrate, packing procedures, etc., prediction of v_{opt} is of little use.

Interestingly, for a 10 μm ODS stationary phase, Hirata and Jinno[15] found the H/v plots for a 0.12 mm i.d. column to be higher-lying than the 0.15 mm ≈ 0.25 mm which were above the 0.20 mm i.d. column which achieved $H_{min} \approx$ 2dp). Importantly, v_{opt} appeared to be independent of column diameter at approximately 0.28 mm s^{-1}. Allowing for the difference in particle size between that study (10 μm) and this one (5 μm), via the observed shift in the optimum linear velocity (v_{opt}) for conventional columns,[10] the expected v_{opt} for our packed capillary column would be <5 mm s^{-1}. With t_o = 2.83 minutes and a 30 cm long column in this study, the standard linear velocity used was 18 mm s^{-1}, thus indicating that the flow rate could be reduced by >3 before v_{opt} was reached. Subsequent experimentation was found to be consistent with this. The conclusions to be drawn from these comparisons are that the mass transfer characteristics of our stationary phase are comparable with those for the better published data[11,15] and superior to others.[10] The quality of the packing procedure cannot be evaluated since H_{min} was not determined.

Rather than determine the value of v_{opt}, the flow rate was decreased to see if the separation was improved, and, when this was seen to be so, a process of trial and error was invoked. The flow rate was initially reduced to maximise separation of the most difficult pair (ST(8) & SP(9)) and then adjusted to try to optimise the rest of the separation. Figure 5 shows the final result for the combined flow (f) and solvent(s) program (p) shown in Table 3. The effect on the front end of the separation is as desired with all compounds separated; including ST, SP and the second SAM hydrolysis product(27). We were unable to extend that quality of separation to the rest of the compounds in a reasonable total analysis time.

It is clear that reductions in the flow rate down to 40% of the standard 6

Table 3

Gradient Program used for the Chromatogram Shown in Figure 6

Time (Minutes)	% 0.001M Phosphate	%MEOH	Pump Flow Rate mL/min
0	100	0	0.4
0.01	95	5	0.4
17	91	9	0.6
30	88	12	0.6
35	82	18	0.4
45	40	60	0.8
50	40	60	0.6
55	40	60	1.0

$\mu L\ min^{-1}$ still yielded increased efficiency. Figure 6 is a plot of N(fsp) versus N(sp) for each of the analytes (mostly) resolved. The solid line corresponds to N(fsp) = N(sp) and no gain in efficiency due to fp. The dashed line is an approximate line of best fit. N(fsp) values were estimated from the chromatogram in Figure 5 for which the combined flow and solvent program is given in Table 3. N(sp) was calculated from the (related) chromatogram obtained without flow programming. (See ref. 1, Figure 6 for the chromatogram.) The solvent(s) only gradient program (p) was 0-10% MEOH at 0.01 minutes, to 12% at 30 minutes, then to 18% MEOH at 35 minutes and to 30% at 40 minutes. Comparison of this program with Table 3 shows that the fsp used a weaker or equal solvent strength up to 35 minutes. After that, it was necessary to utilise a greater solvent strength to achieve a similar total analysis time. However, from 35 minutes plus the delay time of 6.57 minutes and 2.83 minutes of hold-up time (≈ 44½ minutes) or soon afterwards, the effect of the increased solvent strength in the fsp will override the effect of the reduced flow rate and the elution pattern will alter.

In Figure 5, it seems clear that this transition took place before the elution of compound 14. It is only the compounds eluted earlier for which the effect of fp may be isolated. Thus, compounds 1, 2, 3, 5, 27, 6, 7 and 10 are eluted with increased plate counts due to the reduced flow rate, only. Of these, it is also significant that compounds 1, 2, 3 and 10 are among those eluted with the

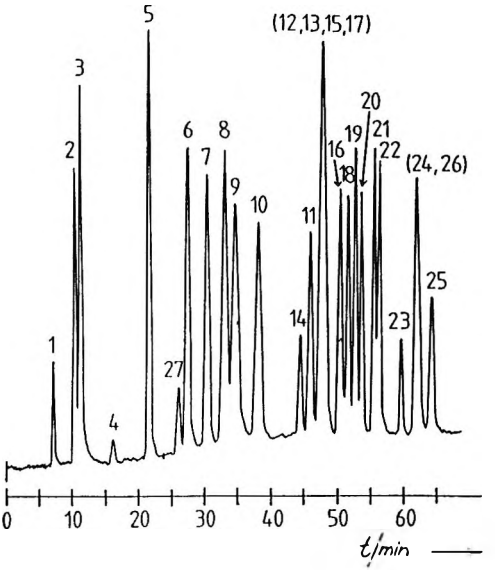


Figure 5. The chromatogram obtained using combined flow and solvent programming as given in Table 3. The numbering of the compounds is as in Figure 1. 27 is the second SAM hydrolysis product.

greatest increase in efficiency relative to the average (dashed line). These are the compounds in the retention time regions that experienced the lowest average flow rates. It is also especially significant for compounds 1, 2, 3, 5, 27, 6 and 7 because of the weaker solvent strength at the start of the fsp. This would lead to relative peak broadening and tend to counter the gain in efficiency due to moving down the N/v curve towards v_{opt}. The magnitude of the increased efficiency due to fp is easy to under-estimate on the log-log scale necessarily used to equally show all of the data. For example, for representative compounds 1, 3 and 5, N(fsp) is 1340, 2070 and 6850 compared with N(sp) of 510, 1150 and 4400, respectively.

CONCLUSIONS

Examination of ACN as an alternative organic modifier to MEOH in the separation of 22 sulphonamides and 3 dihydrofolate reductase inhibitors has revealed some differences in selectivity, particularly in the most difficult part of the separation. In general, however, this was more than countered by losses of

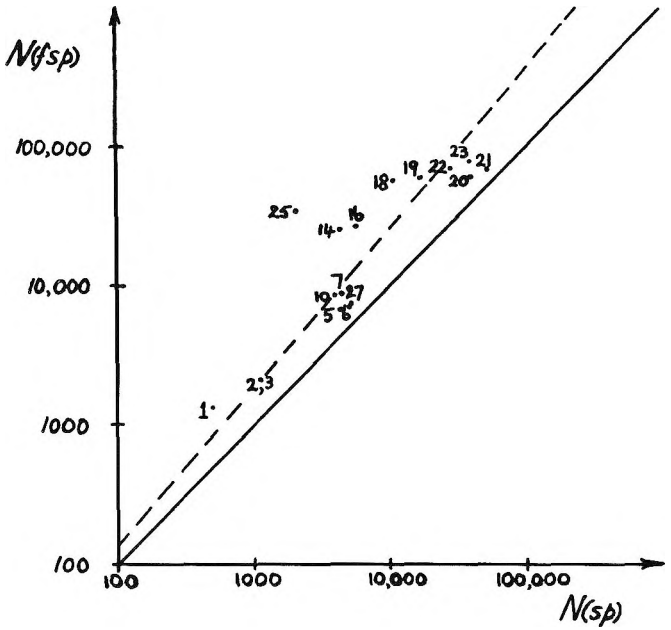


Figure 6. The influence of flow programming on the number of theoretical plates(N) for the compounds (mostly) resolved in the two chromatograms compared. N(fsp) values were estimated from the chromatogram in Figure 6 for which the combined flow (f) and solvent (s) gradient program (p) is given in Table 3. N(sp) was calculated from the chromatogram obtained without flow programming. (See ref. 1. Figure 6 is the chromatogram and the text gives the solvent(s) program(p).) The compounds are numbered as in Figure 1.

resolution due to changed relative retentions in other parts. Attempts to incorporate the benefits of ACN into MEOH gradients were unsuccessful and a complex dependence upon the effects of the two organic modifiers was indicated. On the other hand, concurrent flow and solvent programming clearly demonstrated the positive effect of reduced flow rates and plate heights in the front half of the separation. One of the pairs of drugs most difficult to separate under the majority of conditions investigated are sulphathiazole (ST,8) and sulphapyridine (SP,9). This pair was 2 of the first 13 compounds to be eluted, all of which were resolved. Separation of all 25 drugs simultaneously

may be possible with flow programming, but only with excessively long analysis times.

ACKNOWLEDGEMENTS

We thank the Varian Instrument Division, Walnut Creek, CA, Drs. Terry Sheehan and Rich Simpson for the donation of the LC equipment and columns. MCR thanks the Federal Government for an Australian Postgraduate Research Award.

REFERENCES

1. M. C. Ricci, R. F. Cross, J. Liq. Chromatogr., in press.

2. R. W. Roos, J. Assoc. Off. Anal. Chem., **64**, 851-854 (1981).

3. R. F. Cross, J. Chromatogr., **478**, 423-428 (1989).

4. R. F. Cross, R. L. Cunico, LC Magazine, **2**, 678-673 (1984).

5. L. R. Snyder, J. J. Kirkland, in **Introduction to Modern Liquid Chromatography**, 2nd Ed. Wiley, New York, 1979, pp 169-173.

6. R. F. Cross, J. L. Ezzell, B. E. Richter, J. Chromatogr. Sci., **31**, 162-169 (1993).

7. K. Aizetmuller, J. High Res. Chromatogr., **13**, 375-378 (1990).

8. V. Lesins, E. Ruckenstein, J. Chromatogr., **467**, 1-14 (1989).

9. M. C. Ricci, R. F. Cross, J. Microcol. Sep., **5**, 207-215 (1993).

10. M. W. Dong, R. Grant, LC Magazine, **2**, 294-303 (1984).

11. M. Verzele, C. Dewaele, "Packing Materials and Packing Techniques for Micro-HPLC Columns," in **Microbore Column Chromatography**, F. Yang, ed., Chromatogr. Sci. Series, J. Cazes, Ser. Ed., Vol. 45, Marcel Decker, New York, 1989, pp 50-55.

12. A. M. Krstulovic, P. R. Brown, in **Reverse Phase HPLC**, Wiley, New York 1982, p. 22.

13. G. Guiochon, H. Colin, "Narrow-Bore and Micro-Bore Columns in Liquid Chromatography," in **Microcolumn HPLC**, P. Kucera, ed., J. Chrom. Lib., Vol. 28, Elsevier, New York, 1984, pp 16-24.

14. P. Kucera, J. Chromatogr., **198**, 93-109 (1980).

15. Y. Hirata, K. Jinno, "High Resolution RPLC with a Packed Glass Capillary Column," in **Microcolumn Separations**, M. V. Novotny, D. Ishii, eds., J. Chromatogr. Lib., Vol. 30, Elsevier, New York, 1989, pp 48-49.

Received September 5, 1995
Accepted September 22, 1995
Manuscript 3970

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 565–581 (1996)

SEPARATION AND DETERMINATION OF CHROMIUM(VI), MOLYBDENUM(VI) AND VANADIUM(V) IN STEEL USING CAPILLARY ION ELECTROPHORESIS WITH DIRECT UV DETECTION

Li Jia, Hongwei Zhang, Zhide Hu

Department of Chemistry
Lanzhou University
Lanzhou 730000

ABSTRACT

The application of capillary ion electrophoresis to the separation and determination of chromium (VI), molybdenum (VI) and vanadium (V) with direct on-column UV detection at 254 nm is described. Using a cathodic injection and anodic detection scheme, Cr(VI), Mo(VI) and V(V) were separated in about 4 min in a fused silica capillary column, with a phosphate buffer of pH 12.0, at an applied voltage of 15 KV, followed by direct UV detection. An electro-osmotic flow modifier, namely a cetyltrimethylammonium bromide (CTAB), is necessary to shorten the migration times of these ions. Incorporating citrate into the electrophoretic buffer markedly improves the sensitivity and efficiency.

The influence of several experimental parameters on both sensitivity and efficiency was investigated. Linearity of calibration graphs is observed for about three orders of magnitude with sub-ppm detection limits.

The applicability of the method to the analysis of steel samples is demonstrated. The peak response is strongly dependent upon ionic strength of sample solution and appropriate measurers, such as standard addition, must be used to account for differences in standard and sample conductance.

INTRODUCTION

High performance capillary zone electrophoresis (CZE) is a technique for the separation of charged species in small capillaries at electric field strengths in the range of 10 to 30 KV. It is a highly efficient separation method, capable of yielding excellent resolution of ionized compounds based on the combined effects of electrophoresis and electro-osmosis. The majority of CZE applications have involved the separation of biological macromolecules. However, the technique is amenable to the separation of any ionic species. Although the earliest report of electrophoretic separations of inorganic cations appeared in 1967,[1] the lack of a universal detection scheme for nonchromoplloric analytes hampered early development of separations for inorganic anions. This method has been introduced into inorganic analysis only in the last 3 years.[2-5]

When applicable, the simplest means of monitoring inorganic ions separated by CZE is direct UV absorbance.[6-8] Unfortunately, many ions do not demonstrate significant absorbance at convenient wavelengths and may not be detectable using direct UV detection without complexing agents and a bare fused-silica capillary. A fairly promising approach of overcoming these problems is based on the addition of complexing components to the electrophoretic buffer. These complexing agents can selectively moderate the mobility of metal cations, owing to the formation of metal complexes of different stability within the capillary. This CZE separation mode was first proposed by Foret et al.[9] in combination with indirect UV detection for the separation of lanthanides and then intensively developed by Weston et al.[10]

Another promising possibility for complexation CZE is the complete conversion of metal ions into negatively charged chelates, which can move with different mobilities in the opposite cathode to anode direction. In addition, direct spectrophotometric detection of metal chelates can be performed. Popular ligands for the metal cation separations include cyanide,[11,12] 8-hydroxyquinoline-5-sulfonic acid,[13,14] 4-(2-pyridyazo)-resorcinol,[15] lactate,[16,17] EDTA[18] and α-hydroxyisobutyric acid.[19] According to these solutions, a new separation technique for the analysis of inorganic and organic ions, called capillary ion electrophoresis (CIE) has recently been introduced.[20] This paper

presents a method for the separation and determination of Cr(VI), Mo(VI) and V(V) using direct UV detection by CIE. In this paper, complexing agents only help to improve the sensitivity and efficiency. They do not solve the detection problem nor do they make possible indirect detection.

CIE is a branch of capillary electrophoresis (CE) which is optimized for the rapid analysis of low molecular mass anions and cations. Separations by CIE are based on differences in the electrophoretic mobilities of the injected ions.[21,22] Electrophoretic mobilities are influenced by the structural properties of solutes such as size, shape, Stokes' radius, charge and mass, in addition to the interaction of those solutes with the carrier electrolyte. Each of these variables is affected by properties of the electrolyte, such as pH, ionic strength or viscosity. CIE offers many advantages for the analysis of inorganic and organic acid anions in aqueous matrices. Rapid, highly efficient separations with different selectivities, simplicity and economy are obtained. The technique has been successfully applied to the analysis of a variety of anionic solutes in several complex sample matrices.[23,24]

So far, little attention has been given to studies of the separation and determination of Cr(VI), Mo(VI) and V(V) by CIE. This paper presents a method for the separation and determination of these ions by CIE. The migration behaviour and the optimization of the separation conditions for these three ions, by controlling the electrophoretic buffer parameters, are discussed here. In this paper, both qualitative and quantitative aspects of the application of CIE for the analysis of trace Cr(VI), Mo(VI) and V(V) in moderate ionic strength practical samples are discussed.

EXPERIMENTAL

Instrument

A Waters (Milford, MA, USA) Quanta 4000 capillary electrophoresis system, equipped with a negative high voltage power supply, was used throughout this study, with a Model 820 data station. Polyamide-coated fused-silica capillaries, 51.7 cm total length, 75 μm internal diameter, were obtained from Yong Nian Photoconductive Fibre Factory, Hebei, China. A window for on-column detection was created, 7.3 cm from the end of the capillary. Direct UV detection was accomplished with a Hg lamp and a 254 nm optical filter. Hydrostatic sample mode was selected for injection and sample time was set at

30 s. The separation voltage applied was -15 KV. Data acquisition rate was 20 points/s. The electro-osmotic velocity was measured with formyl amine ($HCONH_2$).

Before each run, a 3 min purge of the capillary with electrolyte was programmed. All operations were at room temperature. All conditional experiments were completed using 10 μg/ml of Cr(VI), 20 μg/ml of Mo(VI) and 10 μg/ml of V(V) (present in the forms of CrO_4^{2-}, MoO_4^{2-} and VO_3^-, respectively).

Reagents

All chemicals were of analytical, reagent grade. All solutions were prepared using filtered, degassed and deionized distilled water.

The phosphate carrier electrolytes were prepared as 6.0 mmol/L solutions containing 2.0 mmol/L of citrate (Beijing Chemical Plant, China) and 0.15 mmol/L of CTAB. The pH of the electrophoretic buffer was adjusted to 12.0 by addition of an appropriate volume of concentrated KOH solution.

Na_2CrO_4, Na_2MoO_4 and NH_4VO_3 were obtained from Beijing Chemical Plant, China. Stock standard solutions of each of these ions were prepared in distilled water at a 1.000 mg/mL concentration. Less concentrated standard solutions were prepared by dilution from the stock solution.

Procedure

At the beginning of each experimental day, it was sufficient to rinse the capillary with deionized water for about 15 min. However, in order to keep the capillary clean, the capillary was flushed with 0.5 mol/L KOH solution after being used three or four days.

Experiments were performed to optimize the CIE system. Unless otherwise specified, the standard conditions used for individual parameters of the system are: -15 KV run voltage, 30s hydrostatic loading time and a working electrolyte consisting of 6.0 mmol/L of phosphate, 2.0 mmol/L of citrate and 0.15 mmol/L of CTAB. Detection was by UV absorbance at 254 nm. Before each run, the capillary is purged for 3.0 min under vacuum.

When doing conditional experiments, the standard sample mixture was

diluted using distilled water. When making a calibration curve (peak area versus concentration of analyte of interest), the standard solutions were diluted using electrolyte buffer (6.0 mmol/L phosphate + 2.0 mmol/L citrate).

Sample Preparation

About 0.5 g of steel samples is weighed accurately and dissolved by HCl and a few drops of HNO_3. Adjust the pH of the sample solution to more than 13 with NaOH. In order to ensure that all of chromium, molybdenum and vanadium in samples can be converted to CrO_4^{2-}, MoO_4^{2-} and VO_3^-, heat the above sample solution as 30% H_2O_2 is dropped into it. Remove the ferric hydroxide precipitate produced in the solution by centrifugation and remove surplus H_2O_2 by heating. Finally, the sample solution is diluted to an accurate volume for determination.

RESULTS AND DISCUSSION

pH of Phosphate Electrolytes

The pH of the electrolyte solution used for separation can have a large effect on the separation of Cr(VI), Mo(VI) and V(V). The effect of pH on the migration times of the three ions can be seen in Figure 1. For the purpose of comparison, experimental conditions such as concentration of phosphate and concentration of CTAB were kept 10 mmol/L and 0.15 mmol/L, respectively. The applied voltage was -15 KV and the temperature was at 22.5 ±1°C.

There are three effects worth noting. Firstly, as the pH is lowered, the migration times increase, due to the decrease in the EOF. Likewise, as the pH is increased, the migration times decrease, due to the increase in the EOF.

The second point of interest is that, in addition to the change in mobility due to the EOF, the pH is also affecting the oxidation states of the three ions. As the pH is decreased, the three ions are easy to condense. There is a balance between CrO_4^{2-} and $Cr_2O_7^{2-}$ in solution. Decreasing pH helps to form $Cr_2O_7^{2-}$. By contrast, increasing pH favors formation of CrO_4^{2-}. MoO_4^{2-} can be condensed to form $Mo_7O_{24}^{6-}$ at pH 6 and $MO_8O_{26}^{4-}$ at pH 1.5-2.9. VO_3^- can be condensed to form $V_2O_7^{4-}$ at ,approximately pH 10.0, $H_2V_4O_{13}^{4-}$ at about pH 9.0 and $H_4V_5O_{16}^{3-}$ at approximately pH. When the pH was below 10.0, the peak of V(V) did not appear in the electropherograms, due to its condensation. The experimental result showed that V(V) was the easiest to condense among the

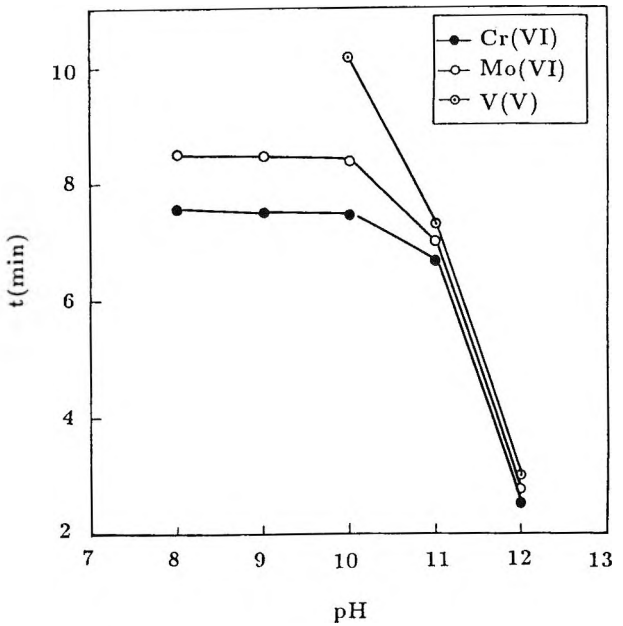


Figure 1. The effect of pH on the migration times of the three ions. When the pH was above 10.0, Cr(VI), Mo(VI) and V(V) were, respectively, present in the forms of CrO_4^{2-}, MoO_4^{2-} and VO_3^-. Later we will discuss the migration order of the three ions in the present work.

Thirdly, the carrier ion-phosphate displays large changes in mobility due to changes in pH. This phenomenon can be seen from the peak shapes. When the pH was at 12.0, the mobility of phosphate was close to the mobilities of the three ions, leading to symmetrical peaks and high efficiency. When the pH was at 12.0, the migration times were shortest for the three ions. If the pH was above 12.0, highly alkaline solutions could change the behavior of the inner surface of the capillary, resulting in poor reproducibility. Owing to the above reasons, 12.0 was selected as the most suitable pH.

Influence of the Concentration of Phosphate

The effect of the concentration of phosphate on the separation behavior of the ions was studied while keeping CTAB at 0.15 mmol/L and pH at 12.0. The ionic strength of electrolyte was mainly controlled by the concentration of phosphate. The ionic strength of the background electrolyte plays three

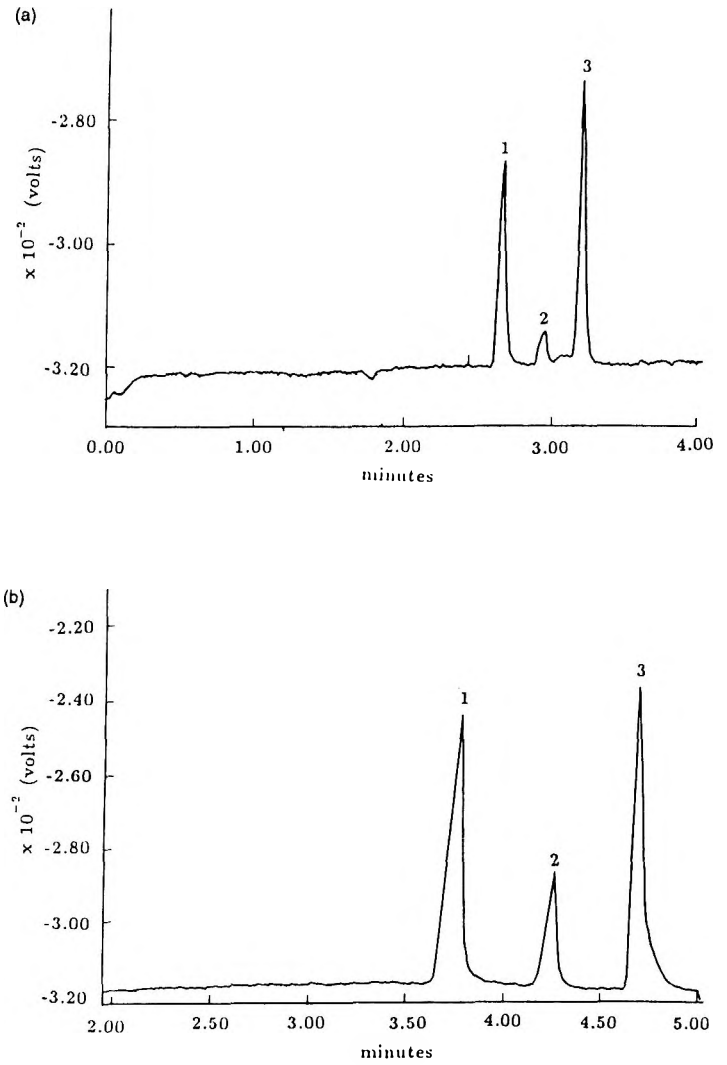


Figure 2. Effect of citrate addition to the phosphate electrolyte on the separation of Cr(VI), Mo(VI) and V(V). Capillary(75 Hm), 51.7 cm length. (a) Electrolyte composed of 6.0 mmol/L phosphate, 0.15 mmol/L CTAB. (b) Electrolyte was same as electrolyte (a), but with 2.0 mmol/L citrate, pH = 12.0, -15 KV applied for separation. Direct UV detection at 254 nm, anode detection. Gravity injection, 10 cm for 30 s. Peaks: 1= Cr(VI) (10.0 μg/mL); 2 = Mo(VI) (20.0 μg/mL); 3 = V(V) (10.0 μg/mL).

different roles in the CZE separations. Firstly, increasing concentration of background electrolyte decreases the EOF. Secondly, it increases efficiency due to higher field strength.[25] Thirdly, it produces higher current, resulting in the inability to effectively dissipate heat. This effect is manifested as noise and baseline aberrations. Taking the three effects into account, we selected 6.0 mmol/L phosphate as optimum concentration, which produces an acceptable current (54.7 μA) to minimize noise while maintaining good peak efficiency.

Influence of the Concentration of Citrate

The influence of citrate addition to the capillary electrophoresis electrolyte was investigated while keeping phosphate at 6.0 mmol/L, CTAB at 0.15 mmol/L, pH at 12.0 and the applied voltage at -15 KV. Figure 2 shows the effect of citrate addition to the phosphate electrolyte system. The second electropherogram in Figure 2 shows the separation of Cr(VI), Mo(VI) and V(V) in an electrolyte similar to that used for the first electropherogram, but with the inclusion of 2.0 mmol/L citrate.

The addition of citrate increases the viscosity and the ionic strength of the electrolyte, resulting in a decrease of electro-osmotic flow. The decrease in EOF results in a small increase in the migration times of the three ions. The addition of citrate causes a change in current due to the increase of ionic strength of electrolyte. But the change in current is not large. The current increased from 54.7 μA at 6.0 mmol/L phosphate to 71.6 μA at 6.0 mmol/L phosphate and 2.0 mmol/L citrate. The change in current was acceptable and did not cause significant increase in baseline noise. It must also be noted that there is a striking increase in efficiency. The efficiency increases with the increase of the concentration of citrate due to the increase of the ionic strength of the electrolyte.

The effect of citrate on the sensitivities of the three ions was also investigated. Figure 3 demonstrates the influence of the concentration of citrate on the sensitivity. In Figure 3, the units for the peak areas are microvolts (corrected for migration times). Since this quantity varies with the zone velocity, the peak areas corrected for migration times were used throughout the report. From Figure 3, we can see, when the concentration of citrate was below 2.0 mmol/L, the peak areas of the three ions increase with the increase of the concentration of citrate, partly due to the decrease in adsorption of the three ions. When the concentration of citrate was above 2.0 mmol/L, the peak areas decrease with the increase of the concentration of citrate. The reason for this

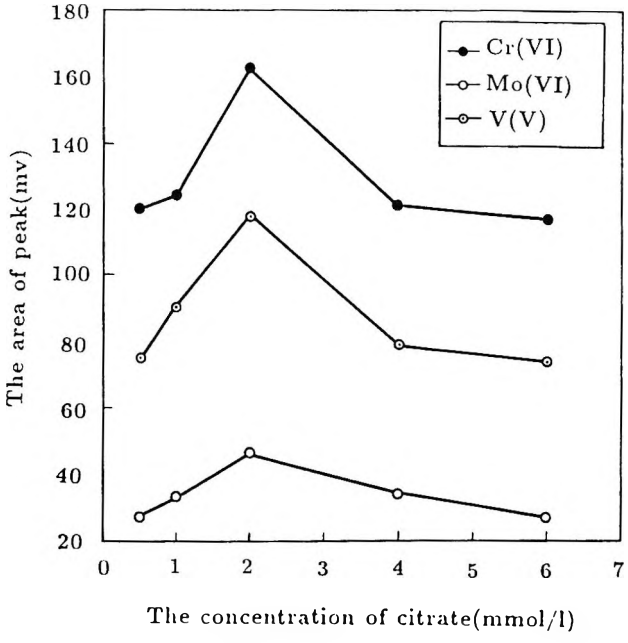


Figure 3. The effect of concentration of citrate on the sensitivities for determination Cr(VI), Mo(VI) and V(V).

observation needs to be explored in greater detail. 2.0 mmol/L of citrate proved to be the best compromise between efficiency, acceptable baseline noise level and sensitivity.

Influence of CTAB

For a normal bare silica capillary, the direction of electro-osmotic flow is from anode to cathode. Cr(VI), Mo(VI) and V(V) are anions under the experimental conditions. A cathode injection and anode detection scheme was used. The three ions migrate from cathode to anode under the applied voltage. In order to minimize the time of analysis, it is desirable to produce an electro-osmotic flow in the same direction as the migration of the analytes. The surfactant CTAB, as an EOF modifier, is used in the electrolyte. CTAB dynamically coats the inner wall of the fused-silica capillary and imparts a positive charge on the wall and changes the EOF towards the anode, thus augmenting the apparent mobility of the three ions. The influence of concentration of CTAB on the migration times was studied. Increasing the

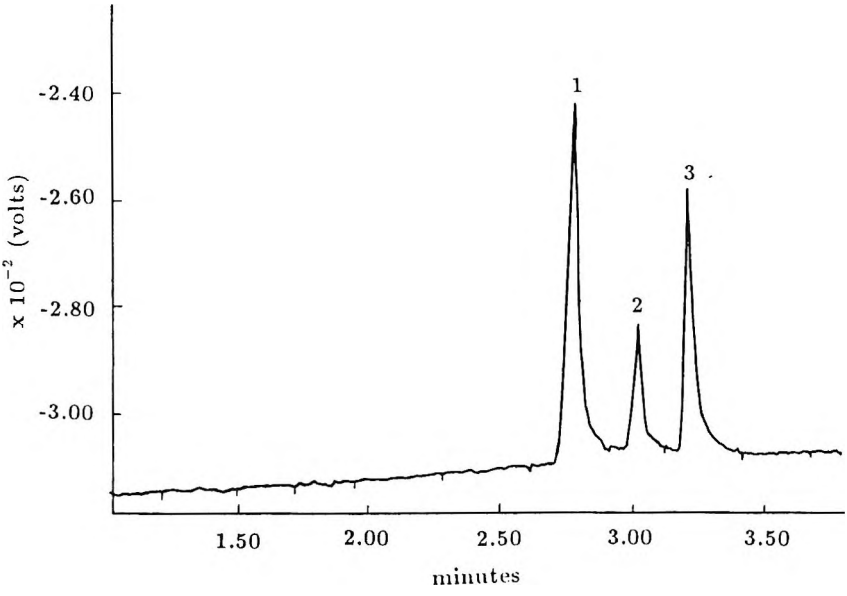


Figure 4. Effect of sodium chloride concentration on the separation of Cr(VI), Mo(VI) and V(V). Experimental conditions: capillary (75 μm), 51.7 cm length; Electrolyte composed of 6.0 mmol/L phosphate, 2.0 mmol/L citrate and 0.15 mmol/L CTAB, pH=12.0; -15 KV applied for separation. Direct UV detection at 254 nm, anode detection. Gravity injection, 10 cm for 30 s.; Peaks: 1 = Cr(VI) (5.0 μg/mL); 2 = Mo(VI) (5.0 μg/mL); 3 = V(V) (5.0 μg/mL)
The mixed standard solution was diluted using 6.0 mmol/L phosphate and 2.0 mmol/L citrate solution.

Table 1

Calibration Data Between 0.5 to 50 μg/mL for the Three Ions

Component	Slope	y intercept	Correlation Coefficient
Cr(VI)	9.88	1.39	0.9998
Mo(VI)	1.29	0.59	0.9997
V(V)	10.4	-0.05	0.9997

The unit of the peak area is microvolts. The unit of C is μg/mL.

concentration of CTAB above 0.10 mmol/L decreases the migration times. Decreasing the concentration of CTAB below 0.10 mmol/L did not appreciably change the magnitude of the reversed EOF, resulting in a little change on the migration times of the three ions. In this paper, 0.15 mmol/L of CTAB was selected.

Quantitation

The usefulness of the method for quantitative monitoring of the three ions was evaluated by means of calibration and reproducibility experiments. In order to study the peak area reproducibility and the variation of the peak areas with concentration, several consecutive runs with different standard solutions were made. As shown in Table I, valid calibration plots could be obtained for the three ions. The precision of five consecutive determinations was evaluated at 5 ppm for the three ions. All analytical conditions were as described in the Experimental section. The results, calculated as relative standard deviations (R.S.D.) of peak areas, along with the concentrations of Cr(VI), Mo(VI) and V(V) in the standards used for the repeat runs, are 4.5%, 7.2% and 3.0% respectively. The detection limits of Cr(VI), Mo(VI) and V(V) achieved at 2 times the signal-to-noise ratio are 0.2 μg/mL, 1.0 μg/mL and 0.2 μg/mL, respectively.

Application

From the sample pretreatment, we know that high concentrations of sodium chloride are introduced into the sample solution. Thus, the sample solution has high ionic strength, which has an effect on the separation behavior of Cr(VI), Mo(VI) and V(V). In order to analyze the amounts of the three ions in the sample, an experiment was performed in which CZE analysis was done with solutions containing a constant amount of Cr(VI), Mo(VI) and V(V), 5, 5, and 5 μg/mL, respectively, and from 10 to 40 mmol/L sodium chloride. Figures 4-8 show the effect of sodium chloride concentration on the migration behavior of the three ions.

From Figures 4-8, we can draw the conclusion that the lower the ionic strength of the sample, the greater will be the enhancement of efficiency and sensitivity, and the shorter the migration times of analytes. When the concentration of sodium chloride is above 20 mmol/L, the Cr(VI) peak is not separated from the Mo(VI) peak. After addition of a constant

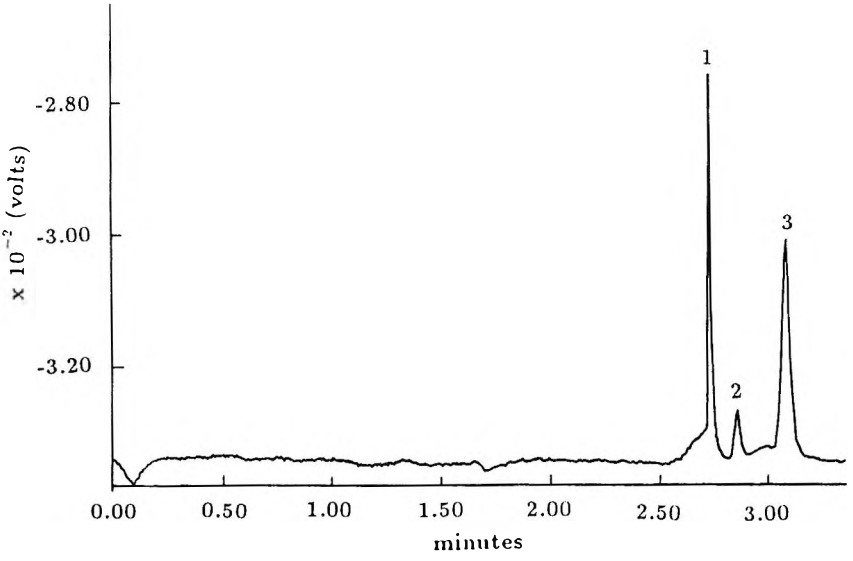


Figure 5. The diluting solution was same as Figure 4, but with 10 mmol/L NaCl.

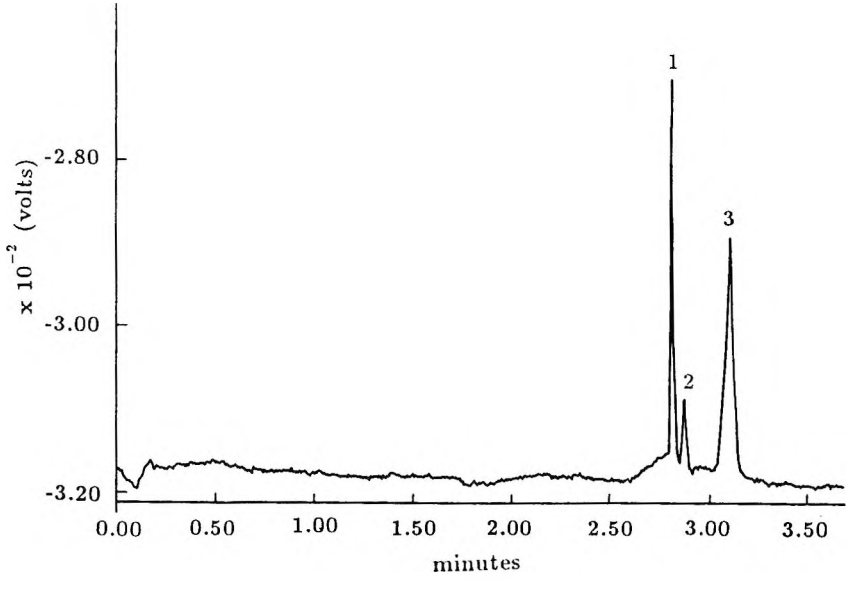


Figure 6. The diluting solution was same as Figure 4, but with 20 mmol/L NaCl.

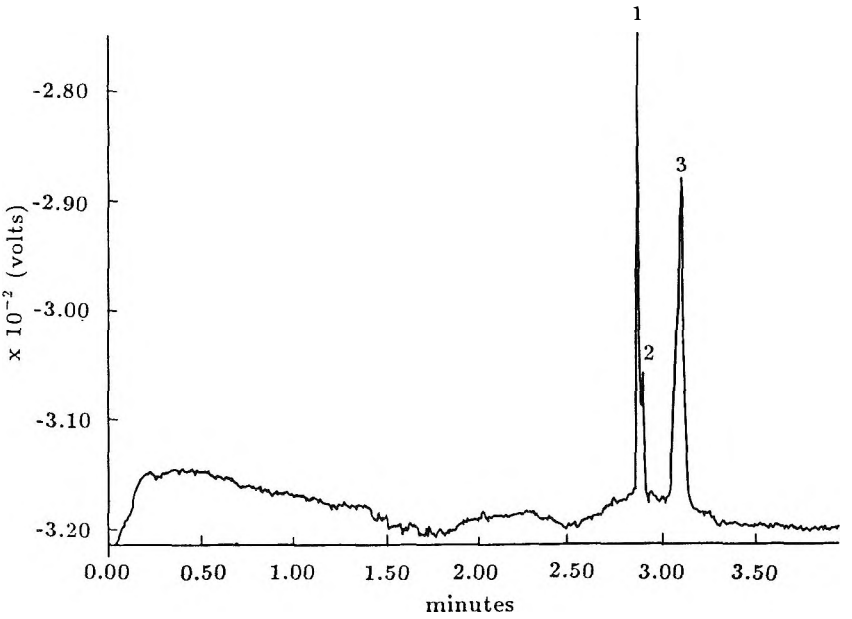


Figure 7. The diluting solution was same as Figure 4, but with 30 mmol/L NaCl.

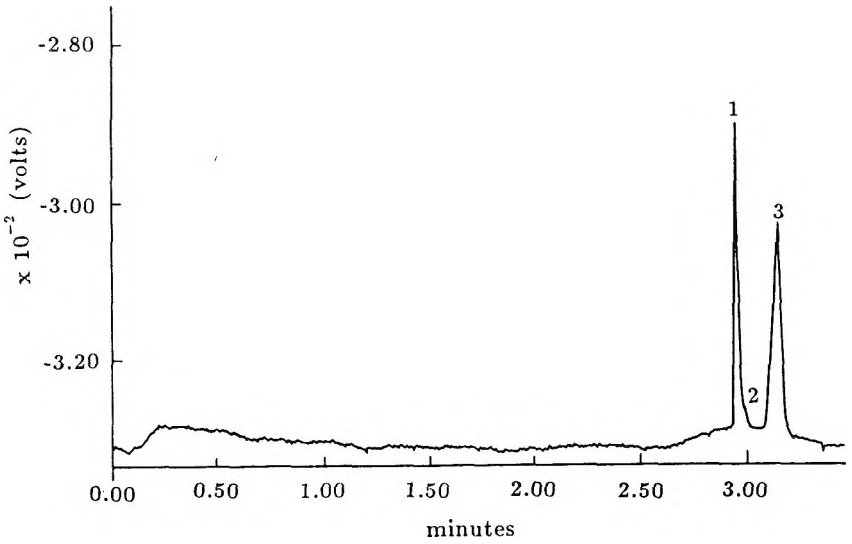


Figure 8. The diluting solution was same as Figure 4, but with 40 mmol/L NaCl.

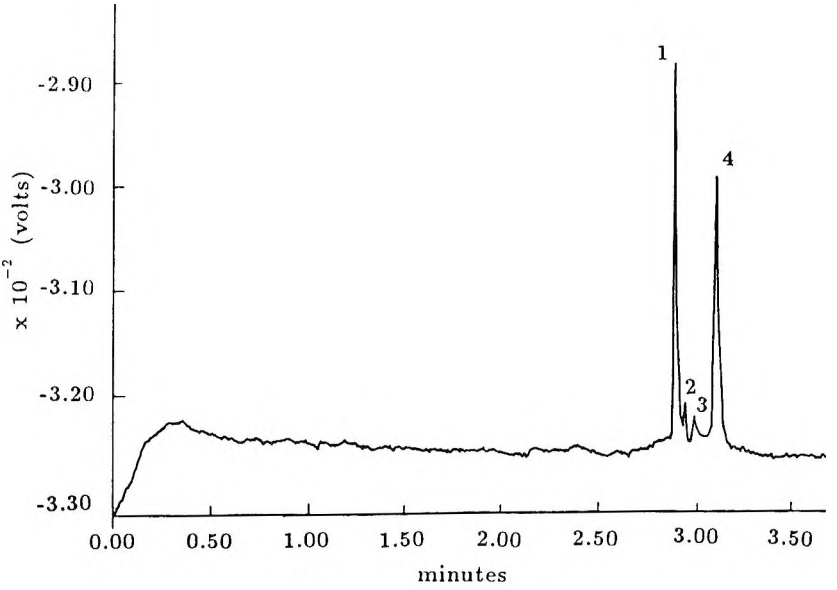


Figure 9. Electropherogram of steel sample solution. Electrophoretic conditions as in Figure 4. Peaks: 1= Cr(VI); 2 = Mo(VI); 3 = Not identified; 4 = V(V).

amount of sodium chloride, lower than 20 mmol/L, the peak area reproducibility and the value of the correlation coefficients of the straight line fits indicate that the approach can be used for quantitative analyses of Cr, Mo and V in steel samples. If the ionic strength of the sample is higher, the approach is less attractive.

In order to analyze the amounts of Cr, Mo and V in the steel sample, the pH of the pretreated sample solution was first adjusted to 12.0 and then several dilutions were made. In this way, the ionic strength of the sample solution was adjusted to be suitable for analysis. According to the distilled sample conductance, adjust the conductance of the standard solutions and make the working curve. Several consecutive electropherograms of the sample were taken.

Figure 9 shows a representative run. The determined values of the concentrations for Cr, Mo and V are in good agreement with the analytical information provided for the standard steel sample.

CONCLUSION

This is the first example of using capillary electrophoresis for the separation of Cr(VI), Mo(VI) and V(V). A major advantage of such a system is its high selectivity, which permits the separation of Cr(VI), Mo(VI) and V(V) in a short analysis time without interference from other ions. The addition of the citrate to the electrophoretic buffer markedly improves the sensitivity and efficiency. The peak response is strongly dependent upon ionic strength of sample solution and appropriate measures, such as standard addition, must be used to account for differences in standard and sample conductance. The approach was successfully applied for analysis of Cr(VI), Mo(VI) and V(V) in a steel sample.

ACKNOWLEDGEMENT

This work was financially supported by the National Natural Science Foundation and the Doctoral Degree Foundation of the State Education Commission of China.

REFERENCES

1. S. Hjerten, Chromatogr. Rev., **9**, 122 (1967).

2. J. Romano, P. Jandik, W. R. Jones, P. E. Jackson, J. Chromatogr., **546**, 411 (1991).
3. L. Gross, E. S. Yeung, Anal. Chem., **62**, 427 (1990).

4. W. Beck, H. Engelhardt, Chromatographia, **33**, 313 (1992).

5. W. Buchberger, P. Haddad, J. Chromatogr., **608**, 65 (1992).

6. M. B. Amran, M. D. Lakkis, F. Lagarde, M. J. F. Leroy, J. F. Lopez-Sanchez, G. Rauret, Fresenius' J. Anal. Chem., **345**, 420 (1993).

7. P. Morin, M. B.Amran, S. Favier, R. Heimburger, M. Leroy, Fresenius' J. Anal. Chem., **342**, 357 (1992).

8. B. Jones, Y. Takahashi, Kankyo Kagaku, **3**, 424 (1993).

9. F. Foret, S. Fanali, A. Nardi, P. Bocek, Electrophoresis, **11**, 780 (1990).

10. A. Weston, P. R. Brown, P. Jandik, W. R. Jones, A. L. Heckenbery, J. Chromatogr., **593**, 289 (1992).

11. W. Buchberger, O. P. Semenova, A. R. Timerbaev, J. High Resolut. Chromatogr., **16**, 153 (1993).

12. M. Agullar, A. Farran, M. Martinez, J. Chromatogr., **635**, 127 (1993).

13. A. R.Timerbaev, O. P. Semenova, G. K. Bonn, Chromatographia, **37**, 497 (1993).

14. A. R. Timerbaev, W. Buchberger, O. P. Semenova, G. K. Bonn, J. Chromatogr., **630**, 379 (1992).

15. N. Ihi, H. Hoshino, T. Yotsuyanagi, Chem. Lett., **4**, 701 (1993).

16. Y. Shiand, J. S. Fritz, J. Chromatogr., **640**, 473 (1993).

17. T. I. Lin, Y. H. Lee, Y. C. Chen, J. Chromatogr., **654**, 167 (1993).

18. S. Motomizu, M. Oshima, S. Matsuda, Y. Obata, H. Tanaka, Anal. Sci., **8**, 619 (1992).

19. M. Koberda, M. Konkowski, P. Youngberg, W. R. Jones, A. Weston, J. Chromatogr., **602**, 235 (1992).

20. W. R. Jones, P .Jandik, Am. Lab., **22**, 51 (1990).

21. J. Jorgensen, K. Lukas, Anal. Chem., **53**, 1298 (1981).

22. J. Jorgensen, K. Lukas, Science, **222**, 226 (1983).

23. D. R. Salomon, J. Romano, J. Chromatogr., **602**, 219 (1992).

24. W. R. Jones, P . Jandik, J. Chromatogr., **608**, 385 (1992).

25. R. Wallingford, A. Ewing, Adv. Chromatogr., **29**, 1 (1989).

Received May 7, 1995
Accepted September 6, 1995
Manuscript 3858

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 583–591 (1996)

HPLC DETERMINATION OF PINDOLOL BENZOATE AND PINDOLOL 2-METHOXYPHENYLACETATE

H. Pietiläinen,*[1] T. Saesmaa[2]

[1] Orion Corporation
Orion-Farmos Pharmaceuticals
P.O. Box 65
FIN-02101 Espoo, Finland

[2] Pharmacy of Mäntsälä
Keskustie 11
FIN-04600 Mäntsälä, Finland

ABSTRACT

In order to investigate the formation of organic salts of drugs, two new salts of pindolol were precipitated using benzoic acid and 2-methoxyphenylacetic acid. The reversed-phase high-performance liquid chromatographic (HPLC) method was developed for the determination of the mole ratio of the salt components. The method permits the simultaneous quantitation of the cationic and anionic parts of the salts prepared. The method was validated with respect to linearity and precision of the chromatographic run and the assay. Also, the precisions at the lowest and the highest levels of the linearity curves were tested. The system suitability was studied by calculating the chromatographic parameters, such as capacity factor (k'), number of theoretical plates (N), tailing factor (T) and resolution (R) between pindolol and organic acids used.

INTRODUCTION

Pindolol is a widely used 13-adrenergic blocker, commercially marketed in pharmaceutical preparations as pure base. It is classified as non-cardioselective and it has intrinsic sympathomimetic actions, but little membrane-stabilising activity. It is used mainly in the treatment of hypertension, cardiac arrhythmias and glaucoma.

Different methods for the quantitation of pindolol are described in the various pharmacopoeias. The most simple method for assaying pindolol is to titrate with hydrochloric acid and to determine the end point potentiometrically.[1,2] Also, spectrophotometric determination at a wavelength of 264 nm has been used.[3] Different reversed-phase HPLC methods have been reported for the quantitation of pindolol. For example, reversed-phase columns with nitrile groups or dimethylsilane chemically bound to porous silica have been used with the mobile phase of pH 5.0.[4] A mobile phase of pH 6.7 has been reported for screening basic nitrogenous drugs.[5]

The HPLC methods have also been used for the simultaneous quantitation of the pharmaceutical salt components in order to find out the mole ratio of the salts.[6-8] The present method was developed by modifying the method reported for separation of nine β-blocking agents.[9]

In order to investigate the formation of organic salts, two salts of pindolol were precipitated using benzoic acid and 2-methoxyphenylacetic acid. The syntheses, elemental analyses and physical studies of the salts formed have been described elsewhere.[10] The present study was undertaken to develop and validate a rapid, simple and precise reversed-phase HPLC method for the determination of the mole ratio of two new salts precipitated.

EXPERIMENTAL

Chemicals

Pindolol (Sandoz Pharm AG, Switzerland) was kindly supplied by Orion-Farmos Pharmaceuticals (Espoo, Finland) and it was Ph. Eur. grade. Analytical grade benzoic acid (Darmstadt, Germany) and 2-methoxyphenylacetic acid (Munchen, Germany) were obtained from Merck. Sodium 1-heptanesulfonic acid 1-hydrate was from Eastman Kodak (Rochester, N.Y., U.S.A) and it was HPLC grade. The methanol used for the mobile phase and sample preparation was of HPLC quality. Uracil (50 ,ug/ml) was used for

the determination of residence time of the unretained compound for the calculation of capacity factors (k').

Chromatographic Conditions

The HPLC consisted of a Hewlett-Packard 1090 (Avondale, PA, U.S.A.) equipped with a diode array UV-VIS detector and a Hewlett-Packard 79994A workstation. Separation of the salt components was carried out using a 5-μm Hypersil RP-18 column (200 x 4.6 mm I.D. stainless steel).

The mobile phase consisted of methanol (pump A) and 2 % acetic acid solution containing sodium 1-heptanesulfonate (1.1 %) as an ion pair (pump B). The elution was carried out with a gradient program by increasing the amount of methanol 6% / min from 20 to 80 % (v/v).

The oven temperature was +40°C and the flow rate 1.5 mL/min. The detection was carried out for pindolol, benzoic acid and 2-methoxyphenylacetic acid at 254 nm, 273 nm and 270 nm, respectively. The volume iniected was 20 μL.

Calibration Graphs

Stock solutions of pindolol (0.9 mg/ml), benzoic acid (0.4 mg/ml) and 2-methoxyphenylacetic acid (0.5 mg/ml) were prepared in methanol. The calibration graphs were examined by performing measurements on five different concentrations diluted from the stock solutions in methanol-water (1:1); concentration ranges were 35-175 μg/mL for pindolol, 15-80 μg/mL for benzoic acid and 20-100 μg/mL for 2-methoxyphenylacetic acid. Each solution was injected twice.

Standard Solution for the Assay

Standard solutions for the assay were made from stock solutions to yield 145 μg/mL of pindolol and 60 μg/mL of benzoic acid (= Standard solution A) and 145 μg/mL of pindolol and 80 μg/mL of 2-methoxyphenylacetic acid (= Standard solution B). Dilutions of stock solutions were made in methanol-water (1:1).

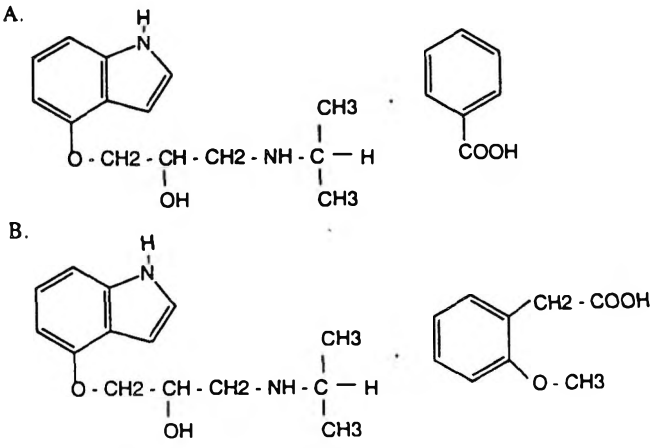


Figure 1. Structure of pindolol benzoate (A) and pindolol 2-methoxyphenylacetate (B).

Sample Preparation

The samples were diluted to a concentration of about 200 μg/mL with a mixture of methanol-water (1:1). The mole ratio of the components of the product was determined by assaying six samples of the salts against the standards.

RESULTS AND DISCUSSION

In order to investigate the formation of organic salts, two salts of pindolol were synthesized. Syntheses and physical studies of the salts have been described elsewhere.[10]

Stuctures of the salts formed are shown in Fig. 1. The compositions of the products were first established by HPLC. For that reason, an appropriate HPLC method was developed.

The HPLC method developed gave a baseline resolution for the cationic and anionic components of the salts (see Fig. 2). The retention times for benzoic acid, 2-methoxyphenyl-acetic acid and pindolol were 5.9, 6.1 and 7.8 min, respectively.

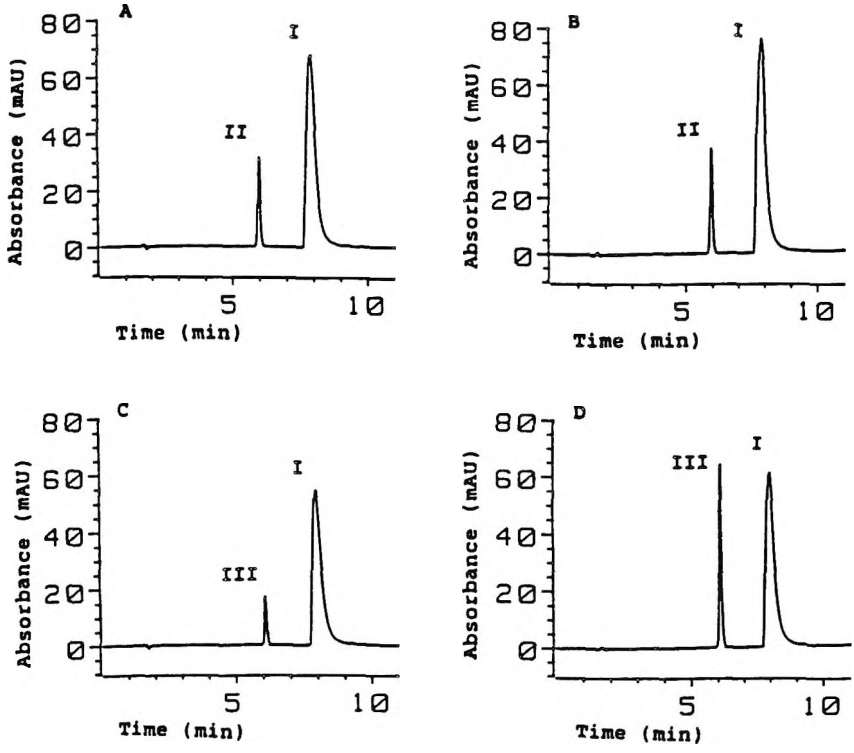


Figure 2. HPLC traces of pindolol benzoate (200μg/mL) at wavelengths 254 nm (A) and 272 nm (B) and pindolol 2-methoxyphenylacetate (200μg/mL) at wavelengths of 254 nm (C) and 270 nm (D).
Peaks: I. Pindolol; II. Benzoic Acid; III. 2-Methoxyphenylacetic acid. For chromatographic conditions, see Experimental section.

Validation of the HPLC Method for the Salt Components

The method was validated with respect to linearity and precision of the chromatographic run and the assay. Also, the precisions at the lowest and the highest levels of the linearity curves were tested.

The system suitability was studied by calculating the chromatographic parameters, such as capacity factor (k'), number of theoretical plates (N), tailing factor (T) and resolution (R) between pindolol and organic acids used.

Table 1

Results of the System Suitability Test in the Method Developed

Std Sol'n	Compound	Precision of the Chrom. Run (RSD %)	Chromatographic Parameter k'	N	T	R
A	Pindolol	0.3	3.8	2600	1.4	
	Benzoic Acid	0.7	2.6	17500	1.0	5.1*
B	Pindolol	0.3	3.8	2600	1.4	
	2-Methoxy-phenylacetic acid	0.1	2.7	18600	1.0	4.7*

Symbols: k' = capacity factor; N = theoretical plates; T = tailing factor; R = resolution. Residence time of an unretained compound (uracil) was 1.6 min (= t_o; used for k' calculations).
* = Resolution of pindolol and benzoic acid
** = Resolution of pindolol and 2-methoxyphenylacetic acid

System Suitability

The system suitability was studied by calculating chromatographic parameters from the chromatograms obtained with the standard solutions A and B containing uracil (about 50 μg/mL). The uracil was used for determining the holdup time (t_o). The data demonstrates that the chromatographic parameters were acceptable (Table 1).

Linearity

The results of the linear regression analyses are described by the equations

$y = 12.05\ x - 4.72$ ($r = 1.0000$) for pindolol,
$y = 3.81\ x - 2.78$ ($r = 0.9999$) for benzoic acid and
$y = 5.44\ x + 0.96$ ($r = 1.0000$) for 2-methoxyphenylacetic acid.

The precisions at the lowest and the highest levels were 0.3 and 0.6 % for pindolol, 0.6 and 0.1 % for 2-methoxyphenylacetic acid and 1.3 and 0.2 % for

Table 2

Determination of Pindolol Benzoate (I) and Pindolol 2-Methoxyphenylacetate (II)

Sample No.	Found (%)							
	I				II			
	Pindolol		Benzoic Acid		Pindolol		2-Methyxyphenyl-Acetic Acid	
	A	B	A	B	A	B	A	B
1	66.5	66.1	32.9	32.8	58.4	59.0	40.1	40.3
2	66.5	65.8	32.9	32.7	58.3	58.4	40.0	40.1
3	66.5	66.2	33.1	32.6	58.6	59.1	40.2	40.4
4	66.4	66.2	32.7	32.7	59.3	59.5	40.1	40.3
5	66.4	66.2	32.4	32.5	59.2	58.6	40.0	39.6
6	66.4	66.4	32.5	32.9	59.1	59.8	40.0	40.5
Mean	66.45	66.15	32.75	32.70	58.82	59.07	40.07	40.20
S.D.	0.05	0.20	0.27	0.14	0.44	0.53	0.08	0.32
R.S.D. (%)	0.1	0.3	0.8	0.4	0.7	0.9	0.2	0.8

A = Precipitation of the salt was made in ethanol.
B = Precipitation of the salt was made in acetone-water (98+2).

benzoic acid, respectively. The data demonstrates that the responses were linear for all salt components and verifies that single point calibration is suitable.

Precision of the Chromatographic Run and the Assay

Precision of the chromatographic run was examined by making six injections from standard solutions A and B. Relative standard deviations for pindolol, benzoic acid and 2-methoxyphenylacetic acid were 0.3, 0.7 and 0.1 %, respectively (Table 1). Precision of the assay was studied by analysing six samples of the salts. Samples were prepared from homogeneous sample material and assayed. Relative standard deviations for all compounds were

under 1.0 % (Table 2). Both the precision of the chromatographic run and the assay were good for all compounds studied.

Determination of Pindolol Benzoate and Pindolol 2-Methoxyphenylacetate

The mole ratio was determined by assaying six samples of the salts versus standards. The mean portions for the salts are shown in Table 2. Those values were close to the theoretical values 67.0 and 33.0 % for pindolol and benzoic acid in pindolol benzoate and 59.9 and 40.1 % for pindolol and 2-methoxyphenylacetic acid in pindolol 2-methoxyphenylacetate, respectively. There was no great difference whether the salt was made in ethanol or in a mixture of acetone and water. This indicates an approximate stoichiometry of 1:1 for both organic salts of pindolol formed. Timolol maleate and metoprolol tartrate also had a 1:1 stoichiometry as determined by elemental analysis.[11,12] Those salts were not, however, quantitated by HPLC.

CONCLUSION

The HPLC method developed for the determination of the mole ratio of the prepared salts permits the simultaneous quantitation of both components of pindolol benzoate and pindolol 2-methoxyphenylacetate. The validation data was acceptable and the results obtained agree with the results from analytical and physical studies[10] of the salts, confirming that two new organic salts of pindolol were formed in the syntheses.

ACKNOWLEDGEMENTS

This study was supported, in part, by the Finnish Cultural Foundation. We also wish to thank Orion-Farmos Pharmaceuticals (Espoo, Finland) for their generous donation of chemicals and for letting us use their modern equipment.

REFERENCES

1. European Pharmacopoeia, Second Edition, Council of Europe, Maissonneuve S.A., France, 1989, p. 634.

2. British Pharmacopoeia, Health Minister, London, 1993, p. 519.

3. British Pharmacopoeia, Health Minister, London, 1993, p. 1061.

4. The United States Pharmacopoeia, XXIII Revision, Pharmacopeial Convention, Rockville, MD, 1995, pp. 1230-1231.

5. R. Gill,"High Pressure Liquid Chromatography," in **Clarke's Isolation and Identification of** Drugs, A. C. Moffat, ed., The Pharmaceutical Press, London, 1986, p.201-220.

6. F. Nachtmann and K. Gstrein, J.Chromatogr., **236**, 461-468(1982).

7. V. Haikala, J.Chromatogr., **389**, 299-305(1987).

8. T. Saesmaa, J.Chromatogr., **455**, 415-419(1988).

9. M. A. Lefebvre, J. Girault, J. B. Fourtillan, J. Liq.Chromatogr., 4**(3)**, 483-500 (1981).

10. H. Pietiläinen, T. Saesmaa and V-P. Tanninen, Eur. J. Pharm.Sci., submitted.

11. D. J. Mazzo, A. E. Loper, "Timolol Maleate" in **Analytical Profiles of Drug Substances,** 1987, Vol. 16, K. Florey, ed., Academic Press, Inc., Orlando, pp. 641-692.

12. J. R. Luch, "Metoprolol Tartrate" in **Analytical Profiles of Drug Substances,** 1983, Vol. 12, K. Florey, ed., Academic Press, New York, pp. 325-356.

Received June 1, 1995
Accepted June 18, 1995
Manuscript 3879

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 593–599 (1996)

FRACTIONATION OF BLACK SEED (*NIGELLA SATIVA* LINN) PROTEINS BY USING ROTOFOR

Afrozul Haq, Nona Remo, Sultan T. Al-Sedairy*

Department of Biological and Medical Research (MBC-03)
King Faisal Specialist Hospital and Research Centre
P.O. Bo 3354
Riyadh 11211 Saudi Arabia

ABSTRACT

Many plants and their products are used as sources of pharmaceuticals and as ingredients of traditional medicines, and are of value in new drug discovery. In this study, we fractionated the whole black seed proteins into twenty fractions by using Rotofor. The pH range was recorded as (2.63 to 10.46). Protein concentration range and percent protein recovered were 0.305 to 1.01 mg/mL and 5 to 17% respectively. A single 4 hr Rotofor run at 12W in the pH 3 to 10 range yielded well separated proteins into twenty fractions. Black seed (*N.sativa*) proteins isolated range between 200 kDa and 14 kDa when these fractions were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

INTRODUCTION

Herbal remedies form a potpourri that ranges from plants that people collect themselves and then take for health reasons to approved medicinal products. Herbal products vary from well known European herbs to less familiar Asian and South American ones and from botanicals with an excellent safety record to potentially dangerous ones, such as broom[1] and yohimbe[2]. The

World Health Organization estimates that some 20,000 species of higher plants are used medicinally throughout the world. A recent compilation on traditional medicines describes the uses of medicinal plants in countries as far apart as China, Ghana, India, Japan, Mexico, Panama, Samoa and Thailand[3].

Nigella sativa (*N.sativa*) commonly known as black seed which belongs to the botanical family of Ranunculaceae, has been in use in many Middle Eastern and Far Eastern countries as a natural remedy for over 2000 years. *N.sativa* seeds are commonly eaten alone or in combination with honey and in many food preparations. Abou-Basha et al,[4] and Aboul-Enein & Abou-Basha,[5] determined thymoquinone, dithymoquinone and thymol in black seed (*N.sativa*) oil by TLC and HPLC. Recently, we successfully studied the effect of water soluble *N.sativa* fractions on lymphocyte response to pooled allogeneic cells from 21 normal donors. *N.sativa* protein extract enhanced the production of interleukin-3 by human lymphocytes when cultured with pooled allogeneic cells or without any aided stimulator. Interestingly, *N.sativa* proteins increased interleukin-1ß, suggesting therefore, that it has an effect on macrophages[6].

Amino acid analysis of the black seed protein hydrolysate by gas chromatography of n-propyl, N-acetyl derivatives showed the presence of 15 amino acids including 9 essential amino acids. The seed protein is rich in arginine, glutamic acid, leucine, phenylalanine and lysine, arginine being the major amino acid[7]. Polypeptides and proteins are playing an increasing role in drug therapy. Molecules such as calcitonin, human growth hormone, interferons, colony stimulating factors have all made an important impact in the market place. A careful evaluation of the published literature leads to the conclusion that under normal circumstances only small quantities of peptides and proteins will be expected to be absorbed intact from the human gastrointestinal tract[8].

In the present study, we fractionated the whole black seed (*N.sativa*) by using Rotofor fractionation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of *N.sativa* proteins.

MATERIALS AND METHODS

Chemicals

Acrylamide, bisacrylamide, TEMED, Biolyte (ampholyte) (Bio-Rad, Richmond, CA, USA). SDS, ammonium persulphate, glycine, Tris (Pharmacia

Biotech, Uppsala, Sweden). NaOH and H_3 PO_4 (Fisher Scientific Co. Pittsburgh, PA. USA) were used.

Protein Extraction

The black seed (*N.sativa* Linn) proteins were defatted by using Soxhlet apparatus. The defatted *N.sativa* was air dried for 24 hr at room temperature and then powdered. Protein concentrate was made according to the method of Mattil[9].

Defatted black seed powder was dissolved in phosphate buffer saline (PBS) in a ratio of 1:10 (w/v) for 1 hr with continuous stirring at room temperature and then centrifuged at 10,000 rpm for 30 min. The supernatant was collected, and protein concentration was determined[10]. Whole black seed proteins were first dialyzed (overnight at 4°C) against 0.01M Tris using 3,500 MW cut off dialysing bags. Following dialysis, carrier ampholyte (Bio-Rad , Bio-Lyte), pH range 3-10, 2% V/V was added and the total volume was brought to 60mL with PBS (pH7.1). Constant power (12 W) was applied to Rotofor for 4 hr with a system cooled to 4°C. Runs were terminated when the voltage had stabilized (1500V) for about 30min.

Twenty Rotofor fractions were collected. Eighteen of these fractions were then analyzed by SDS-PAGE. pH gradient, % protein recovery and total protein concentration in each sample was determined. Removal of ampholyte was accomplished by dialyzing all fractions overnight against 100 volumes of 0.1M Tris, pH 7.4 containing 0.25 mM NaCl and 0.5 mM MgCl2.

Preparative SDS-PAGE

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS) was performed in 12% gels according to Laemmli[11]. Protein samples were added to sample buffer and then denatured by boiling for 5-10 mins. Low molecular weight and high molecular weight calibration kits (Bio-Rad, Richmond, USA) containing myosin (200 kDa), ß-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and lysosyme (14 kDa) were used as marker proteins. Staining of gels was done with R250 coomassie brilliant blue (0.1%).

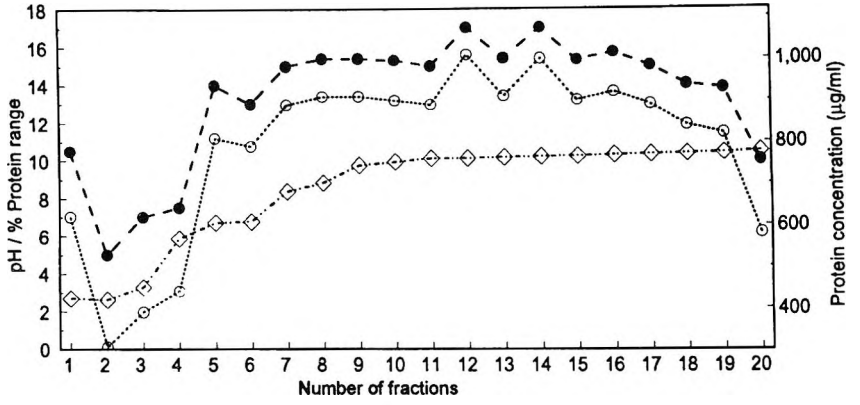


Figure 1. Analysis of 20 Rotofor fractions by pH gradient, percent protein recovered and protein concentration (µg/mL). X-axis represents number of fractions, Y axis represents pH gradient, % of protein range and protein recovery (µg/mL). (◇)pH gradient; (○)% Protein recovery); (●) Protein concentration (µg/mL). X axis - represents number of fractions. Y axis - represents pH gradient/% of protein range and protein recovery (µg/mL).

RESULTS AND DISCUSSION

Rotofor IEF cell provides a rapid and efficient means of purifying proteins without subjecting them to ionic detergents. After 4 hr run at 12 W constant power at 4°C, 20 fractions were collected. Figure 1 shows the pH profile, % of protein recovered and total protein concentration for each fraction. A total of 160 mg (27mL) of *N.sativa* protein was applied to the Rotofor cell and the recovery range was 5 to 17%. Figure 2 shows the results of SDS-PAGE electrophoresis of 18 fractions obtained from Rotofor. Lane A and lane B represent high and low molecular weight marker proteins respectively.

The purpose of fractionating black seed proteins is to use these preparations to raise antibodies in experimental animals *in vivo* and antioxidant defense mechanism *in vitro* that can be classified as a promoter of positive health by combatting free radicals and reactive oxygen species mediated degenerative changes. Although the exact physiological role of these black seed proteins remains uncertain, they all have been shown *in vitro* experiments to affect lymphocytes, macrophages and monocytes[6]. Chromatographic and spectroscopic techniques of isolation, purification and structure determination are highly sensitive and there has never before been a time when a combination of chemical, biological and molecular biological techniques could be used for

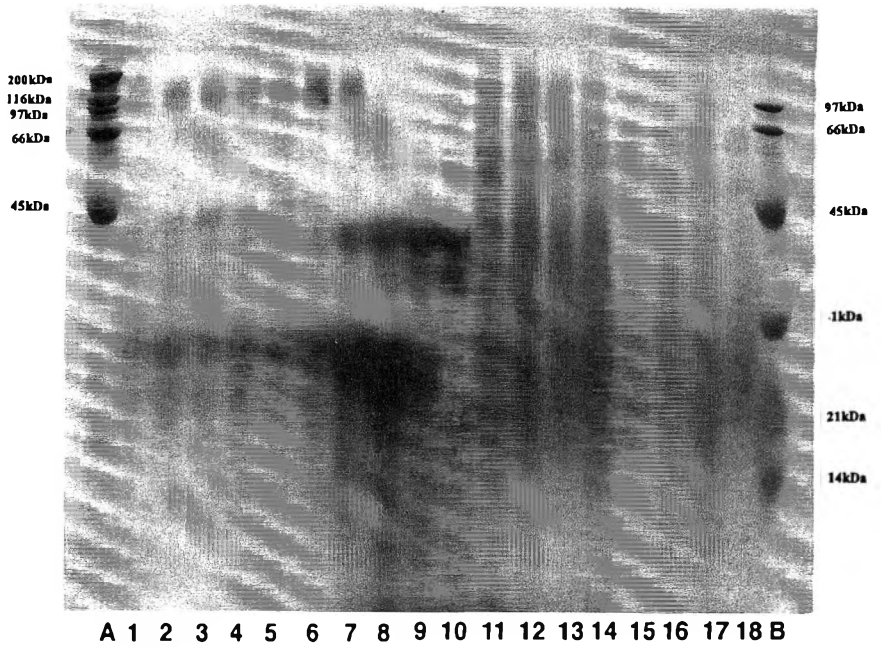


Figure 2. Rotofor compartment fractions 1-18 containing black seed (*N.sativa*) proteins analyzed by SDS-PAGE. Lane A and lane B represent high and low molecular weight markers respectively.

the isolation and production of novel drugs. Such studies offer a logical approach for new drug discovery and for the identification of template molecules for the design of new drug molecules. To our knowledge, we present the first data about the fractionation of black seed proteins. Studies are underway in our lab to isolate and purify the specific protein/peptide molecule(s) from *N.sativa* which may be used as an immunomodulator. On the other hand a number of studies have been conducted with black seed oil *in vivo* and showed that intravenous administration of volatile oil of black seed to rats decreased the arterial blood pressure and the heart rate in dose dependent manner[12]. These authors also found a dose-dependent increase in the respiratory rate and intratracheal pressure when black seed volatile oil was injected (I.V.) to guinea pigs[13].

N.sativa proteins have great potential as functional agents in modulating the immune responses and in many food preparations perhaps as supplements in the diet as antioxidant. However, *N.sativa* proteins can not be recommended

until other studies including nutritional properties, toxicities, antinutritional components, determine its suitability for humans, such studies are underway and will be published separately.

ACKNOWLEDGEMENTS

We sincerely thank Dr. Hassan Y. Aboul-Enein for his critical evaluation of the manuscript and Ms Jennifer M. Almazan for her execellent secretarial assistance and typing the manuscript.

REFERENCES

1. J. H. Galloway, K. Farmer, G. R. Weeks, I. D. Marsh, A. R. W. Forrest, "Potentially Hazardous Compound in a Herbal Slimming Remedy," Lancet, 340-179 (1992).

2. P. A. O.M. De Smet, O. S. N. M. Smeets, "Potential Risk of Health Food Products Containing Yohimbe Extracts," B.M.J. **309**,958 (1994).

3. H. Wagner, N. R. Fransworth, (eds.); **Economic and Medicinal Plant Research**, vol. 4. Plants and Traditional Medicine, London, Academic Press (1990).

4. L. I. Abou-Basha, M. S. Rashed, H. Y. Aboul-Enein, "TLC Assay of Thymoquinone in Black Seed Oil (*Nigella Sativa* Linn) and Identification of Dithymoquinone and Thymol.," J. Liq. Chromatogr. **18(1)**, 105-115 (1995).

5. H.Y. Aboul-Enein, L. I. Abou-Basha, "Simple HPLC Method for the Determination of Thymoquinone in Black Seed Oil (*Nigella Sativa* Linn)," J. Liq. Chromatogr. **18(5)**, 895-902 (1995).

6. A. Haq, M. Abdullatif, P. I. Lobo, K. S. A. Khabar, K. V. Sheth, S. T. Al-Sedairy, "*Nigella sativa*: Effect on Human Lymphocyte and Polymorphonuclear Leukocyte Phagocytic Activity," Immunopharmacol., In press (1995).

7. V. K. Babayan, D. Kootungal, G. A. Halaby, "Proximate Analysis, Fatty Acid and Amino Acid Composition of Nigella Sativa L. Seeds," Inst. Food Technol., 1314-1319 (1978).

8. B. Testa, E. Kyburz, W. Fuhres, R, Giger, (Eds.); In Perspectives in Medicinal Chemistry. (S. S. Davis), **Oral Delivery Systems for Peptides and Proteins**, Verlag Helvetica Chimica Acta, Basel, pp. 533-544 (1993).

9. K. F. Mattil, "Compositional Nutritional and Functional Properties and Quality Criteria of Soy Protein Concentrates and Soy Protein Isolates," J. Am. Oil Chem. Soc., **15**, 18A (1974).

10. M. M. Bradford, "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizling the Principle of Protein Dye-Binding," Anal. Biochem., **72**,248-254 (1976).

11. U.K. Laemmli, "Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T_4," Nature **227**,680-685 (1970).

12. K. E. H. El-Tahir, M.M. S. Ashour, M. M. Al-Harbi, "The Cardiovascular Actions of the Volatile Oil of the Black Seed (*Nigella Sativa*) in Rats: Elucidation of the Mechanism of Action," Gen. Pharm. **24**, 1123-1131 (1993).

13. K. E. H. El-Tahir, M. M. S. Ashour, M. M. Al-Harbi, "The Respiratory Effects of the Volatile Oil of the Black Seed (*Nigella Sativa*) in Guinea Pigs: Elucidation of the Mechanism(s) of Action," Gen. Pharm., **24**, 1115-1122 (1993).

Received July 31, 1995
Accepted August 22, 1995
Manuscript 3944

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 601–610 (1996)

DETERMINATION OF PIPERACILLIN IN BIOLOGICAL SAMPLES BY HPLC

A. Zarzuelo, F. G. López, M. Santos, J. M. Lanao

Department of Pharmacy and
Pharmaceutical Technology
Faculty of Pharmacy
University of Salamanca
37007 Salamanca, Spain

ABSTRACT

A rapid, specific and reliable technique has been developed for the determination of piperacillin in biological samples by High Performance Liquid Chromatography.

To do so, reverse phase liquid chromatography was used, employing acetonitrile-phosphate buffer 0,1M and pH=6 as the mobile phase (20:80 v/v) and a detector wavelength of 254 nm. Concentrations ranged between 0,5 μg/mL and 200 μg/mL, divided into two calibrations: high concentrations, 15 μg/mL to 200 μg/mL, and low concentrations, 0,5 μg/mL to 15 μg/mL.

The intraday and interday reproducibility of the analytical technique was studied by calculating the variation coefficient of 5 samples analyzed each day and on five consecutive days by ANOVA. The coefficients obtained were 4,02% and 7,11%, respectively, for concentrations ranging between 15-200 μg/mL and 5.71% and 7,54% for concentrations between 0,5 and 15 μg/mL.

INTRODUCTION

Piperacillin is an antibiotic belonging to the β-lactam family and is a semisynthetic penicillin that keep the efficiency of its structural analogs and is also active against new groups of organisms from all evolutionary levels.[1]

In clinical practice the drug has been used successfully in a large variety of situations, including sepsis, pneumonia, intra-abdominal infections, bone and soft tissue infections and gynecological and urinary infections. Its activity against *P.aeruginosa* is striking.

Currently, piperacillin is used in combination with aminoglycoside antibiotics owing to their proved synergism. Use of this combination is particularly important for the treatment of systemic infections due to different species of *Pseudomona*, where the clinical activity of carbenicillin is marginal, and for different types of *Enterobacteriaceae*. Another important aspect of the combination is the potential protective effect of piperacillin against the nephrotoxicity elicited by prolonged treatment with aminoglycoside antibiotics.[2,3]

The aim of the present work was to set up a rapid, specific and reliable analytical technique to determine piperacillin in biological samples by HPLC, both in plasma and in tissues such as renal cortex and medulla.

MATERIAL AND METHODS

Reagents

Sodium piperacillin (Lederle); Sodium Cloxacillin (Antibióticos Farma); HPLC grade acetonitrile (Merck); Trichloroacetic acid (Merck); Potassium Hydroxide (Panreac); Monopotassium phosphate (Panreac); Chloroform (Carlo Erba).

Equipment

Kontron liquid chromatograph, mod.420; Kontron UV detector, mod.430; Kontron MT-1 data treatment station; Crison pH-meter, mod.2001; Reverse phase column (RP-18) with 5 μm particle size, a length of 15 cm and an i.d. of 0,4 cm; Heraeus mod.Labofuge 6000 ultracentrifuge; Selecta ultrasound bath,

mod.513; Supelco vacuum system, mod.5-8068 with 0,45 μm Millipore filters; Super Mixer tube shaker; Nitrogen chamber; Thermostatted sand bath (Kowwel T-1).

Chromatographic Conditions

The mobile phase was composed of a mixture of acetonitrile-phosphate buffer 0,1M adjusted to pH=6 with 19N potassium hydroxide (20:80 v/v). This mobile phase was prepared daily and filtered through a Millipore filter with a 0,45 μm pore diameter and was degassed in an ultrasound bath for 15 min prior to use. Flow rate during the assays was 2 mL/min; detector wavelenght was 254 nm;[4] response time was 0,5 sec., and sensitivity was 0,02.

Sample Preparation

Before the samples were injected into the chromatograph, protein denaturing and precipitation were carried out. Sample treatment was as follows:

One hundred μL of a solution of 0,4 mg/mL of cloxacillin in water -used as internal standard- was added to 150 μL of biological sample. The mixture was vortexed for 30 sec., after which 100 μL of a solution of 10% trichloroacetic acid in water -responsible for protein denaturing- was added and the mixture was vortexed again for 30 sec. and then centrifuged for 5 min. at 3.500 r.p.m. The supernatant was directly injected into the chromatograph with a 200 μL fixed-volume loop.

Tissue concentrations are often below 0,5 μg/mL and hence, although the technique becomes more complex, it is necessary to extract the samples with chloroform and then concentrate them to increase the sensitivity limit. In this case, sample treatment was as follows:

One hundred twenty μL of trichloroacetic acid was added to 1 mL of the problem sample -tissue homogenate in Sörensen's buffer, pH=7,4- and this was vortexed for 30 sec. and then centrifuged for 5 min. at 3.500 r.p.m. Following this, the supernatant was collected, adding 5 mL of chloroform. This mixture was vortexed for 1 min. and centrifuged for 10 min. at 3.500 r.p.m. Then collecting the aqueous phase, on which the same operation was repeated, the oil phase of the both previous steps was brought to dryness in a nitrogen chamber

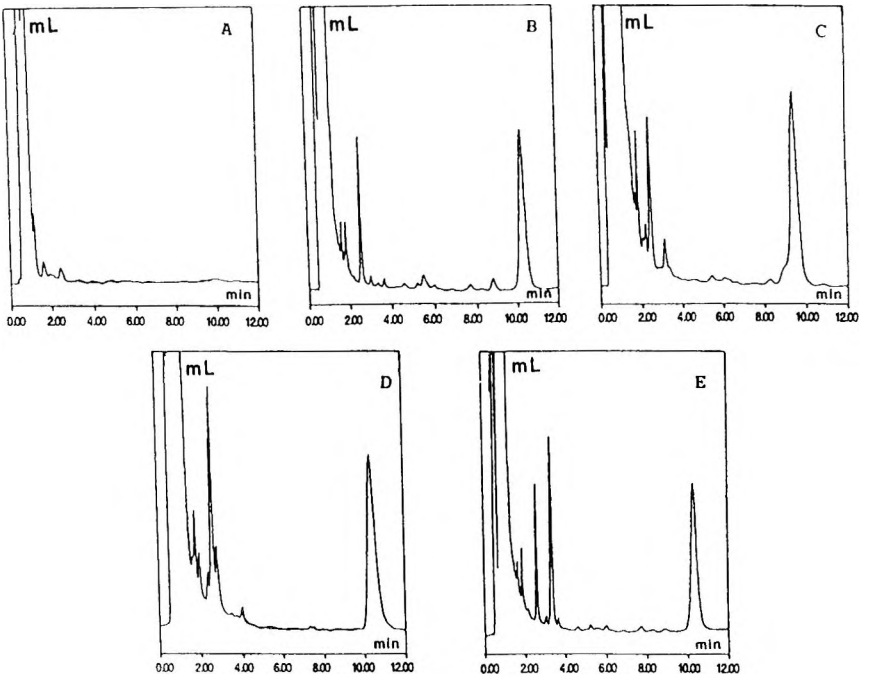


Figure 1. Chromatograms of piperacillin and cloxacillin.

at 37°C, injecting the dry residue dissolved in 250 μL of mobile phase into the chromatograph.

Quantification

The concentration of piperacillin in the problem samples was determined from the following equation:

$$C = (R-A)/B$$

where C is the concentration of piperacillin in μg/mL, A is the ordinate at the origin of the calibration straight line, B is the slope of the calibration line, and R is the peak height ratio (height of piperacillin/height of cloxacillin).

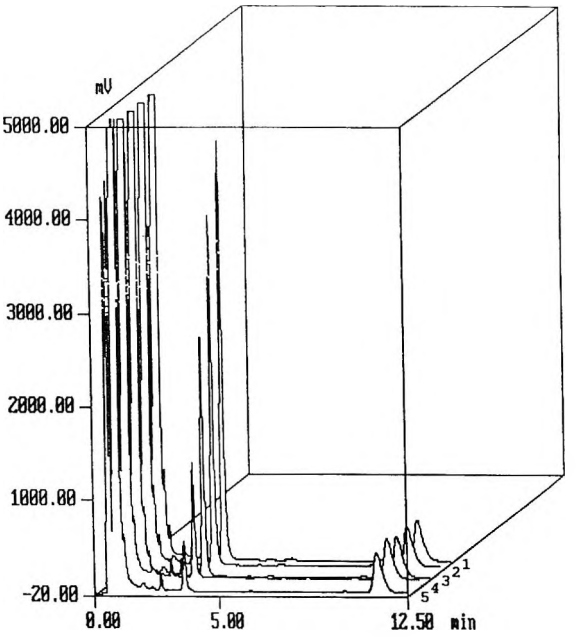


Figure 2. Chromatograms corresponding to the calibration range of piperacillin in plasma:
1. 200 μg/mL; 2. 150 μg/mL; 3. 100 μg/mL; 4. 50 μg/mL; 5. 15 μg/mL

RESULTS AND DISCUSSION

Figure 1 shows the chromatogram of a tissue blank (A), of a plasma blank with internal standard -0,4 mg/mL- (B) and the chromatograms of piperacillin and cloxacillin in different biological samples (C: cortex, M: medulla, E: plasma). Figure 2 included the whole plasma calibration range of piperacillin using the technique.

Under the above-described conditions, piperacillin and cloxacillin were well separated, their retention times being 3,20 and 10,27 min., respectively.

The linearity of the chromatographic technique can be seen in Figures 3 and 4, corresponding to straight line calibrations for high (200-15 μg/mL) and low (15-0,5 μg/mL) concentrations, respectively, of piperacillin in plasma. A linear relationship was established between the peak height ratio (height of piperacillin/ height of 0,4 mg/mL cloxacillin) and the plasma concentration of

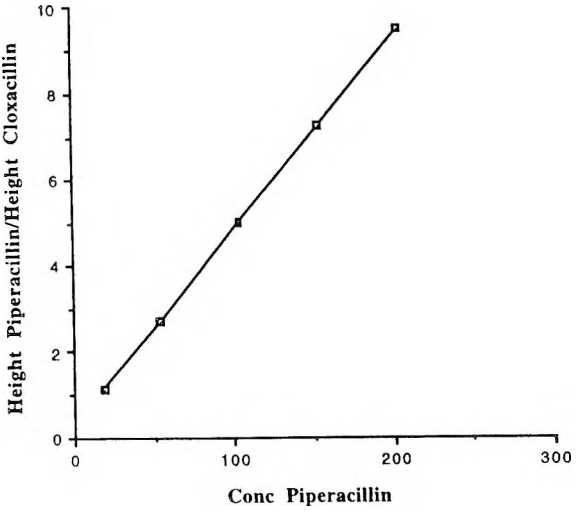


Figure 3. Calibration straight line of piperacillin in plasma for concentrations ranging from 15 to 200 μg/mL.

piperacillin. In both cases, the linear correlation coefficient was 0,999 and regression analysis of the data afforded the following equations:

200-15 μg/mL: $Y = 0,0436 + 0,0579X$
15-0,5 μg/mL: $Y = 0,0045 + 0,0412X$

where X and Y are the peak height ratio and piperacillin concentration, respectively.

Statistical Treatment

To study the reproducibility of the analytical technique, its variation coefficients were studied -both intraday and interday- with an ANOVA Test. To do so, five calibration straight lines were analyzed on 5 consecutive days. The data obtained with the ANOVA Test are shown in Tables 1a & 1b.

The resulting variation coefficients were 4,10% for the intra-day study and 7,11% for the inter-day study. A similar study was conducted for low piperacillin plasma concentrations (15-0,5 μg/mL), the variation coefficients

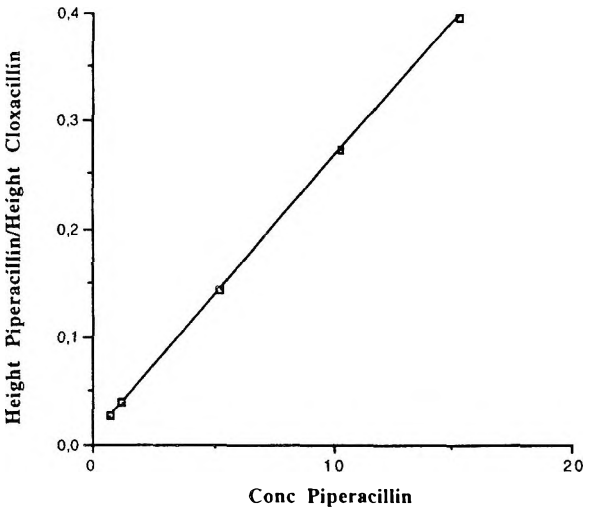


Figure 4. Calibration straight line of piperacillin in plasma for concentrations ranging from 0,5 to 15 μg/mL.

being 5.71% and 7,54% for the intra and inter-day studies, respectively.

CONCLUSIONS

The analytical technique described here is simple and shows good linearity and reproducibility, with a detection limit below 0,5 μg/mL. The technique permits the determination of piperacillin concentrations in biological samples using a small sample volume, which is important when carrying out studies on this type of sample.

All this means that the technique can be routinely used in pharmacokinetic studies with this drug, which are increasingly frequent owing to the importance of the combination of piperacillin with aminoglycosides drugs for the treatment of diverse infections.[5]

With minor modifications, the technique could also be used for the analysis of other β-lactam antibiotics.

Table 1a

Intra and Inter-day ANOVA

Day/Assay	1	2	3	4	5
1	14.75	16.94	16.80	16.79	14.53
2	14.80	14.95	14.80	13.59	13.45
3	15.22	16.38	16.21	13.23	13.89
4	14.67	14.99	15.45	16.53	16.69
5	15.04	16.93	15.04	15.04	15.80
Mean 15.23 ± 0.98 μg/mL					
1	50.89	49.98	52.95	46.34	55.05
2	47.19	49.10	46.47	51.15	47.46
3	48.44	51.04	54.78	50.15	47.77
4	48.85	51.04	54.78	50.15	47.77
5	49.45	50.40	49.31	50.55	49.55
Mean 49.26 ± 2.36 μg/mL					
1	99.87	103.21	104.47	102.23	102.42
2	103.18	105.17	97.34	103.31	102.22
3	98.37	98.11	104.31	103.39	104.06
4	95.15	101.26	98.84	98.42	104.02
5	104.84	100.35	99.11	100.10	103.85
Mean 101.61 ± 2.71 μg/mL					
1	151.20	151.20	149.31	154.41	155.56
2	152.53	147.57	146.82	154.64	149.19
3	143.71	153.02	150.12	149.36	148.20
4	150.51	152.53	153.35	148.22	149.39
5	152.78	149.06	147.48	148.30	149.75
Mean 150.21 ± 2.91 μg/mL					
1	198.10	205.61	196.57	203.23	201.37
2	199.83	194.94	199.42	194.76	205.64
3	206.59	206.36	204.51	194.92	201.87
4	196.66	206.36	201.53	195.61	197.22
5	203,58	189,01	188,59	201,61	199,85
Mean 199.81 ± 4.12 μg/mL					

Table 1b

Test ANOVA

Intraday	**SS**	**MS**	**DF**
Regression	4.904E+02	4.962E+02	1.000E+00
Error	2.211E+00	9.612E+02	2.300E+01
Total	4,926E+02		
Interday			
Regression	1.866E+03	1.883E+03	1.000E+00
Error	1.685E+01	1.719E-01	9.800E+01
Total	1.883E+03		

ACKNOWLEDGEMENTS

Some of the results presented here were obtained with a grant. Project: DGICYT-PB92-0274.

REFERENCES

1. M.F.Parry, S. J. Pancoast, **Antimicrobial Therapy**, Raven Press, Inc., New York, 1984. pp.197-207.

2. T. Hayashi, Y. Watanabe, T. Yasuda, I. Saikawa, J. Katahira, T. Kumada, K. Shimizu, Antimicrobial Agents and Chemotherapy, **32(6)**, 912-918 (1988).

3. W. E. Uber, R. C. Brundage, R. L.White, M. R. Brounley, The Annals of Pharmacotherapy, **25**, 357-359 (1991).

4. D. Debruyne, J. Ph. Ryckelynck, B. H.Ligny, M. Moulin, Journal of Pharmaceutical Sciences, 79, 99-102 (1990).

5. A.Kucers, M. Bennett, **The Use of Antibiotics**, Heinemann, Inc., London, 1979. pp.171-181.

Received July 23, 1995
Accepted August 16, 1995
Manuscript 3945

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 611–624 (1996)

HIGH PERFORMANCE REVERSE PHASE LIQUID CHROMATOGRAPHIC METHOD FOR TOPOTECAN TRACE ANALYSIS

L. C. Hsu

Corporate Environmental Research Laboratory
SmithKline Beecham Corporation
King of Prussia, PA 19406, USA

ABSTRACT

A highly sensitive and selective reversed-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection for topotecan (SK&F 104864A) trace analysis is presented. Topotecan is the prototype of a novel class of antitumor agents, which exert their activities exclusively by inhibition of cellular topoisomerase I. The analytical method is based on an efficient HPLC separation using a Zorbax® StableBond® narrow bore (150 x 2.1 mm i.d.) C_8 column (particle size 5 mm). A secondary-retention phenomenon was noticed during the studies, however, and the problem was solved by adding 0.1% (v/v) triethylamine. Zorbax® StableBond® columns represent the new class of silane-modified silica, and have proved to be stable at least four months with continuously analyzed samples containing bleach and hydrogen peroxide. Column effluents were monitored via fluorescence with the excitation wavelength set at 225 nm and the emission wavelength at 520 nm. The limit of quantification (LOQ) for topotecan was 0.2 ng/mL. Linear response was observed for concentration of topotecan from 0.2 to 20 ng/mL. The method not only provided sensitive, precise and accurate support to photolysis, biodegradation and deactivation treatment system studies but also

reduced the organic solvent waste by two-thirds as compared to conventional HPLC columns (4.6 mm i.d.).

INTRODUCTION

Topotecan ((S)-9-Dimethylaminomethyl-10-hydroxy-camptothecin hydrochloride SK&F 104864A, Fig. 1), is a semisynthetic analogue of camptothecin. Topotecan demonstrated considerable cytotoxic and antitumour activity by exclusive inhibition of topoisomerase I.[1,2] Topoisomerases are intranuclear enzymes that transiently break and rejoin DNA strands to facilitate processes like replication, recombination and transcription that are essential for cell survival. Currently, one SmithKline Beecham facility has been modified to handle secondary production for topotecan clinical trials. Due to its potential health hazards, a topotecan deactivation treatment system was required to treat the waste generated from the packaging process. This system was set up prior to the sewer discharge system and consisted of eight UV lamps with an auxiliary hydrogen peroxide delivery system. Photolysis and biodegradation pathways have also been investigated. To support the large-scale waste treatment system, biodegradation and photolysis studies, an efficient HPLC method capable of analyzing more than three samples per hour was required.

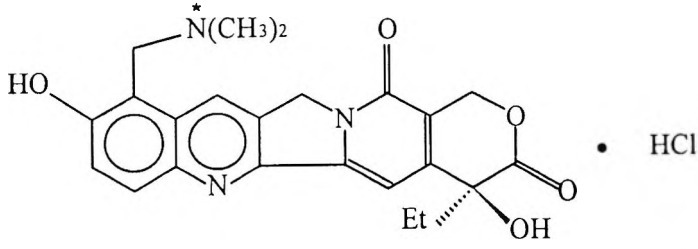


Figure 1. Topotecan (SK&F 104864A) structure. • is the possible adsorption site for silanol groups.

This paper describes an efficient, sensitive and specific HPLC method for the determination of topotecan. By using narrow bore columns (2.1 mm i.d.) with fluorescence detection, the limit of detection and the limit of quantification were found to be at 0.06 ng/mL and 0.2 ng/mL, respectively. This method is also mass spectrometry compatible and, as such, would be suitable for further planned LC-MS investigations.

EXPERIMENTAL

Instrumentation

All measurements were made with a Hewlett-Packard 1050 HPLC system with an HP 1046A fluorescence detector (Hewlett Packard, Palo Alto, CA, USA). The excitation wavelength was set at 250 nm (2 x 2 mm slit) and emission wavelength was set at 520 nm (4 x 4 mm slit). The PMT gain was set according to the sample concentration range and the Xenon lamp flash frequency was set at 200 Hz and the response time was set at 1 sec. A Nelson 900 Series interface and PE Access*Chrom VAX chromatographic software (Perkin-Elmer Nelson System, Cupertino, CA, USA) were used for data collection and analysis. The sampling rate was 1 pt/s and the samples were injected at least three times. Chromatographic separations were carried out on a 150 x 2.1 mm i.d. Zorbax C_8 column (MacMod, Analytical Inc., Chadds Ford, PA, USA) at room temperature. The injection volume was 10 μL. Triethylamine (J. T. Baker, Phillipsburg, NJ, USA) 0.1 % (v/v), adjusted to pH 3 with trifluoroacetic acid (J. T. Baker, Phillipsburg, NJ, USA), was used as mobile phase A and acetonitrile (J. T. Baker, Phillipsburg, NJ, USA) as mobile phase B. The gradient profile was 8% B isocratic for 1 minute; to 35% B in 6 minutes; back to 8% B in 1 minute; and re-equilibrate for 10 mins. Total run time was 18 minutes. The optimal excitation and emission wavelengths were determined by trapping the sample in the flow cell, and the best illumination wavelength was found by scanning the wavelength characteristic of the flow cell contents.

Reagents and Solutions

Mobile phase A was prepared by adding 10 mL triethylamine (TEA) to 970 mL HPLC water followed by pH adjustment to 2.9-3.1 through addition of about 15 mL trifluoroacetic acid (TFA). The final volume was brought to 1000 mL with HPLC water. The solution was mixed and filtered through a separate 0.4-0.5 mm polycarbonate, PVDF, or equivalent membrane filter. A 100 μg/mL topotecan stock solution (SmithKline Beecham Pharmaceutical, King of Prussia, USA) was prepared by accurately weighing about 10 mg of topotecan into a 100 mL volumetric flask. Ten mL's mobile phase A was added to totally dissolve the compound, and then solution was diluted to volume with mobile phase A. The standard solution was stored at 4°C and protected from light.

Standard dilutions of the stock solution were prepared, as needed, using volumetric glassware and mobile phase A.

RESULTS AND DISCUSSION

Narrow Bore Columns and Column Characteristics

Reducing organic solvent waste and the associated disposal cost has become a priority for many HPLC users, especially when the organic waste is potentially hazardous as in the present study. The feasibility and desirability of using capillary zone electrolysis (CZE) in the biodegradation studies of a pharmaceutical compound has been previously demonstrated.[3] With no organic solvent consumption, CZE is an environmentally friendly method for the assay analysis. However, CZE lacks the sensitivity needed for the trace analysis, although laser-induced fluorescence detection has been used with CZE.[4-6] Unfortunately, these lasers are available in quantity only for visible and near-IR wavelengths. Thus, an alternative method was needed for topotecan trace analysis.

Columns with internal diameters of less than 4.6 mm, (i.e., 3.0 mm or 2.1 mm) will dramatically reduce solvent consumption and solvent waste, because a smaller flow rate is used (0.2-0.4 mL/min). Simpson and Brown[7] have compared the performance characteristics, such as column efficiency, selectivity, resolution and detection limit obtained in the narrow bore mode with those obtained by conventional HPLC. There are two additional benefits to use columns with smaller internal diameters.

First, there is an increase in sensitivity. Samples are eluted in smaller peak volumes on 2.1 mm i.d. columns. Therefore, the concentration of the analyte is greater and sensitivity is increased (assuming same injection volumes). Second, narrow bore columns are compatible with most HPLC instruments with little or no system modification; they can also interface directly to other detectors. The lower flow rates (0.2-0.4 mL/min) of small bore columns make it possible to interface directly with many current LC/MS systems. As suggested by Snyder et al.[8] gradient elution was also used to improve peak shape should 10 μL injection cause any noticeable losses in resolution on a 2.1 mm i.d. column. The combination of gradient elution and the narrow bore column provided enough separation power to positively identify topotecan from other possible interference compounds (Fig. 2).

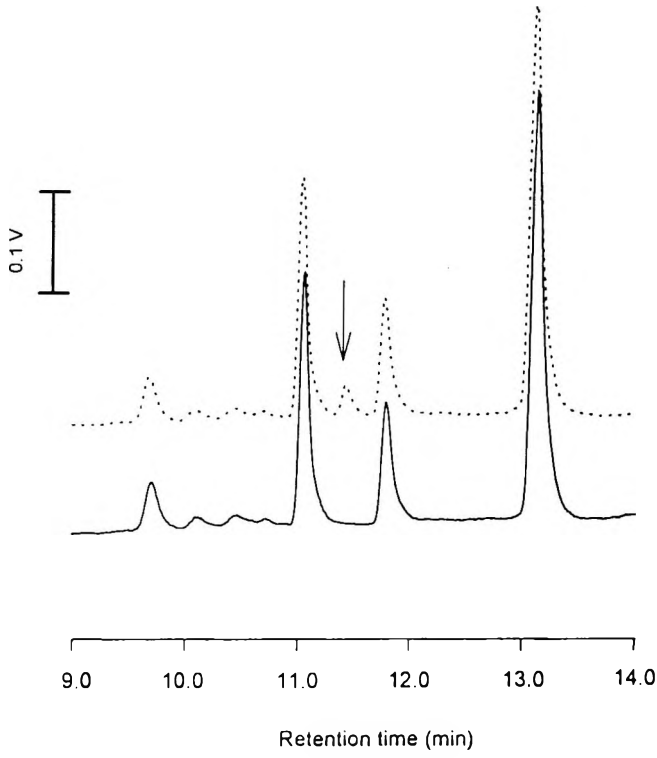


Figure 2. Chromatograms of treated waste samples. Solid line is the treated waste sample chromatogram; dotted line is the same sample spiked with a 2 ng/mL topotecan standard (arrow). HPLC condition: Injection volume: 10 mL; Column temperature: Ambient; Instruments: Hewlett-Packard 1050 with 1046A fluorescence detector; Mobile phase A: 0.1 % TEA and TFA pH 3 solution. Mobile phase B: acetonitrlle. Gradient profile: 8% B isocratic for 1 minute; to 35% B in 6 minutes; and back to 8% B in 1 minute; re-equilibrate for 10 mins.; Detection: fluorescence (ex =225 nm, em=520 nm PMT gain =16).

Zorbax StableBond C_8 columns used in this study represent the new type of siliane bond stationary phases which have been prepared with monomeric "sterically-protected" bonded silanes containing diisopropyl- and diisobutyl-silane side groups and a variety of interactive ligands (C_8, C_{18}, etc.)[9,10] It is the steric bulk of the side group (from dimethyl to diisopropyl) which greatly

Table 1

System Suitability Report During Four Months Studies

Parameter	k'[a]	R[b] (x10^6)	N[c]	A_s[d]
Month 0	4.44	1.96	69295	1.41
Month 1	4.44	2.01	68957	1.40
Month 2	4.57	2.04	70120	1.35
Month 3	4.49	2.02	66453	1.45
Month 4	4.43	1.94	65413	1.46
Average	4.47	1.99	68048	1.41
%R.S.D.[e]	1.32	2.11	2.95	3.12

[a] Capacity Factor; [b] Response Ratio;
[c] Theoretical Plate Number, calculated usign Foley-Dorsey approximation.[14] [d] Asymmetry Factor = ratio of the distances from the perpendicular to the rear side and the front side of the peak, along the 10% horizontal line. [e] % RSD = (Standard deviation (σ) divided by the average) x 100.

increases the stability of these monomeric silane coatings against degradation during use and maintains high column efficiency and reproducibility.[11,12] The columns' characteristics have been reviewed elsewhere.[9-13] The Zorbax StableBond columns appeared to be very stable during the course of our experiments (Fig. 3). Table 1 is the system suitability report during the four months of studies. Although the peak is broader than desired, which may be due to 10 μL injections used to increase the sensitivity, the assymmetry factor is still acceptable (<1.6). It is very important, once the desired separation method has been obtained, that column characteristics should remain unchanged for as long as possible so that any need for further adjustment of separation conditions (or replacement of the column) is minimized.

Secondary-Retention

A secondary-retention phenomenon was observed during the topotecan method development (Fig. 4A). Secondary - retention in the reverse phase

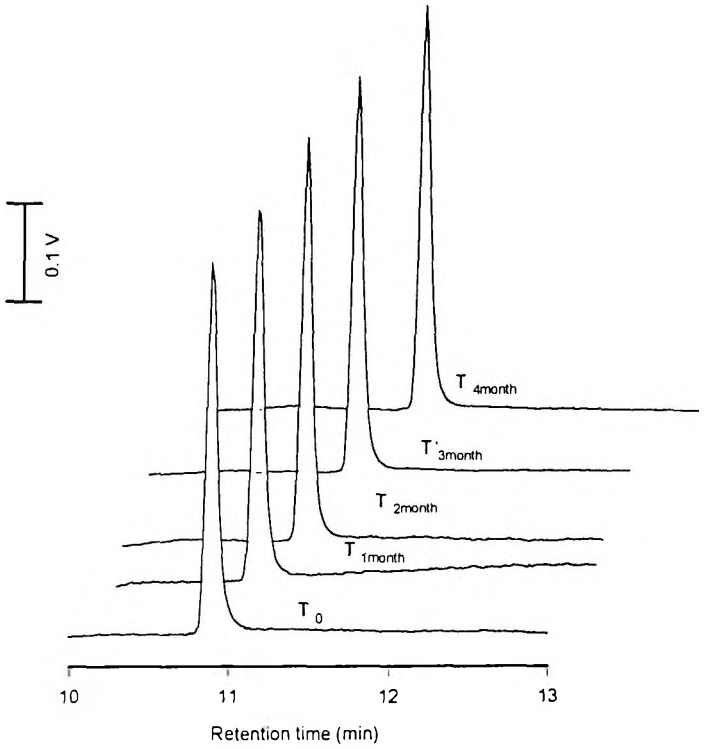


Figure 3. Chromatogram of 20 ng/mL topotecan standard during four months treatibility studies. HPLC conditions are the same as Fig. 2.

separations has been variously attributed to silanol groups of different kinds and to the presence of trace metal impurities in the column packing.[15,16] It is believed that this strong retention in reverse phase systems is due to two kinds of silanol interactions: hydrogen bonding or ion exchange. The benzyldimethylamine functional group (* in Fig. 1) with a pKa of 10.5 [17] is likely the main contributor to the secondary-retention with silanol groups. When a secondary effect such as that of Fig. 4A is present, the most effective solution generally is the addition of some mobile phase modifier that will preferentially interact with (and block) these secondary-retention sites (silanol groups in the present example). Amine-additives such as triethylamine (TEA) are commonly used for this purpose.[18] Fig. 4B shows the effect of adding 0.1 %

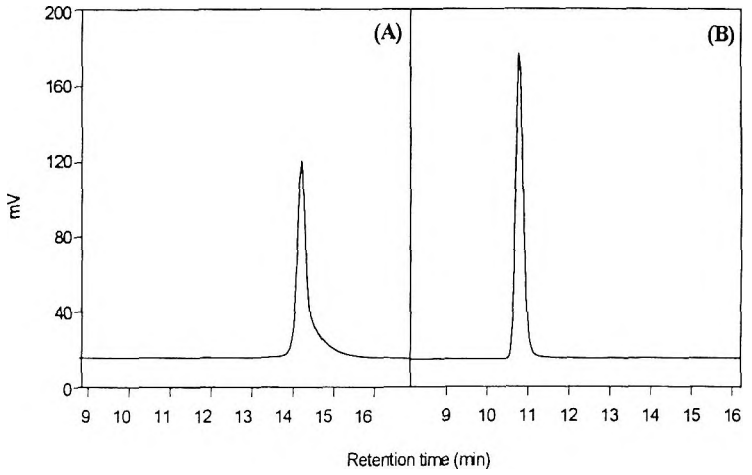


Figure 4. Secondary retention. Topotecan 10 mg/mL in (A) phosphate pH 3 buffer (B) 0.1 %(v/v) TEA/TFA. HPLC conditions are the same as Fig. 2, except fluorescence PMT gain=11. See text for details.

TEA (v/v) to the mobile phase. The result is a pronounced sharpening of the bands for topotecan, with the elimination of any band-tailing. Presumably, the amine-modifier (TEA) effectively eliminates the silanol groups as sorption sites for sample molecules.

Even with the special efforts using the bulky sterically protecting groups in the bonded phase, which have been shown to increase column efficiency and better peak symmetry for basic samples,[13] Zorbax StableBond columns still suffered from the secondary-retention effect. Secondary-retention not only affects the peak shape, but the retention time as well (Fig. 4).

Two interactions are involved in the retention process: from the stationary phase and from the silanol groups. The strong retention caused by secondary-retention will also affect the accuracy of the computer-assisted HPLC method development package such as DryLab® (LC Resources Inc., Walnut Creek, CA) where a single retention is assumed. Currently, the secondary-retention effects on the reverse phase LC retention predictions are under study in our laboratory.

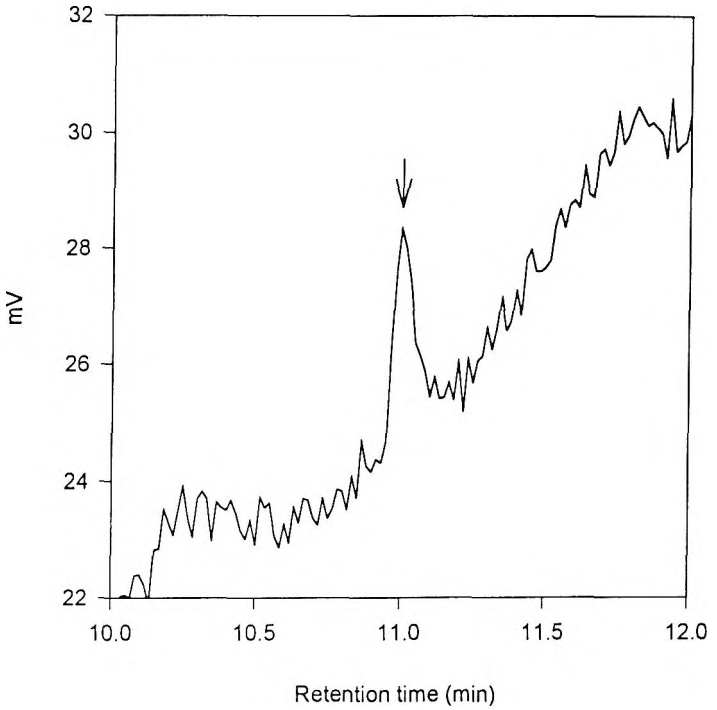


Figure 5. Chromatogram of 0.2 ng/mL topotecan standard. HPLC conditions are the same as Fig. 2.

Sensitivity, Accuracy, Precision and Linearity

The limit of detection (LOD) was determined using Equation 1, derived by Foley and Dorsey:[19]

$$LOD = 3s_B/S \quad (1)$$

where s_B and S are the standard deviation of the noise and the analytical sensitivity (or calibration factor), respectively. The analytical sensitivity is defined as the slope of the calibration curve (signal output per unit concentration). Based on these calculations, the LOD for topotecan is 0.06 ng/mL. The limit of quantification (LOQ) is defined as the amount of analyte

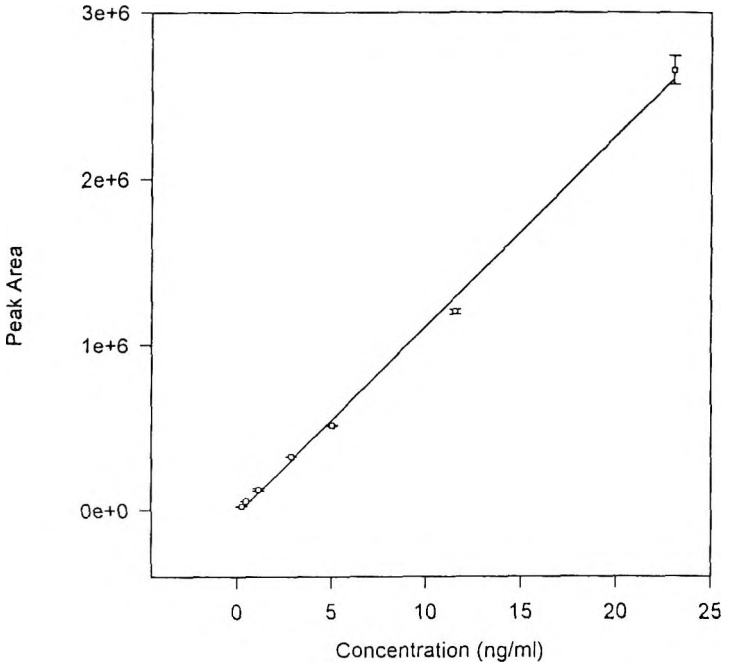


Figure 6. Representative topotecan calibration curve from 0.2 to 20 ng/mL; The error bar is the 95% confidence interval.

detected at ten times the standard deviation of the noise[20] and was calculated using Equation 2:

$$LOQ = 10s_B/S \qquad (2)$$

Using this calculation, the LOQ for topotecan is 0.2 ng/mL. Figure 5 is the chromatogram of 0.2 ng/mL topotecan standard; the signal to noise ratio (S/N) was estimated at 6. An extensive method validation was performed. The within-day precision of the assay was less than 11% for all concentrations within the standard curve range (Table 2). By using 2.1 mm i.d. HPLC columns, the lowest concentration of topotecan that could be determined quantitatively in 1 mL of waste samples was 0.2 ng (475 fmol). The calibration curve obtained was linear over the range of 0.2-20 ng/mL of topotecan. Linear regression analysis of calibration curves provided the equation $y = 1.019 \times 10^{5} x - 5 \times 10^{-3}$ and a correlation coefficient greater than 0.999 (Fig. 6). The

Table 2

Accuracy and Precision Data for Topotecan in Four-Day

Parameter	Concentration (ng/mL)							
	0.2	**0.5**	**1**	**2**	**5**	**8**	**10**	**20**
R.S.D. (%)								
Day1	9.30	4.72	1.89	1.99	0.72	0.70	0.61	1.17
Day2	13.93	2.34	3.77	0.92	0.65	0.57	1.13	1.42
Day3	12.83	4.03	4.58	2.71	2.06	0.64	1.20	1.88
Day4	5.46	5.97	6.04	2.82	1.51	0.84	1.17	0.63
Error (%)[a]								
Day1	10.60	4.52	-5.16	-2.65	-2.20	-5.19	-2.21	-0.29
Day2	10.40	11.48	-2.17	-1.60	-4.07	-4.24	-3.76	0.37
Day3	3.70	0.76	-3.92	-4.22	-3.47	-3.82	-2.13	0.65
Day4	-3.80	3.72	-3.92	-6.72	5.03	-4.36	-2.92	1.97
Day-to-day R.S.D.[b]	13.24	5.78	4.48	2.99	1.73	0.87	1.39	1.58
Within-day R.S.D.[c]	10.38	4.26	4.07	2.11	1.23	0.69	1.03	1.28
Mean Accuracy (%)	92.90	94.9	96.2	96.2	96.3	95.6	97.2	99.0

[a] (Calculated concentration-actual concentration)/actual concentration x 100
[b] Coefficients of variation of daily means
[c] Mean of the daily R.S.D.'s

calibration curves were highly reproducible and the accuracy, estimated by the average concentration back calculated from the composite standard calibration curve, was within 10% of the original value at each concentration. The precision, as measured by the R.S.D.s at each concentration, was within 7% across the calibration range (except for 0.2 ng/mL).

Table 2 summarizes the results obtained from a four-day validation study in which five replicate standards at eight concentrations, 0.2, 0.5, 1, 2, 5, 8, 10 and 20 ng/mL, were analyzed each day. The mean accuracy of the assay at these concentrations ranged from 92.9 to 99%, whereas the within-day precision, indicated by the mean of the daily R.S.D.'s, varied from 0.69 to 10.38%. The reproducibility of the assay was high with day-to-day precision, indicated by the R.S.D.'s of the daily means, ranging from 0.87 to 13.24%.

CONCLUSIONS

A simple and sensitive HPLC method is presented for the trace analysis of topotecan. The assay can detect 0.2 ng/mL and is compatible with many LC/MS interfaces. The increase in the use of narrow bore columns is largely due to several inherent advantages relative to conventional HPLC, such as smaller sample requirements, reduced mobile-phase consumption, and lower costs. With little or no hardware modification, narrow bore columns are the preferable columns for HPLC trace analysis. Although, larger injection volumes (>20 μL) can be used to increase the sensitivity on 4.6 mm id. columns, this usually results in peak broadening, thus, poor reproducibility and resolution. During the four months topotecan treatment analysis, most of the waste samples contained hydrogen peroxide and bleach; Zorbax® StableBond® columns were still very stable as indicated in Table 1. Further work with the LC/MS, for possible topotecan degradants characterization, is currently underway in our laboratory.

ACKNOWLEDGMENTS

The author will like to thank Drs. D. Orvos, J. Brum and Mr. R. Hannah for their help in preparing this manuscript and the support from the management of the Corporate Environmental Research Laboratory.

REFERENCES

1. M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail, G. A. Sim., J. Am. Chem. Soc., **88**, 3888 (1966).

2. M. H. Beijnen, Pharm Weekbl. Sci., **14**, 258 (1992).

3. L. C. Hsu, D. R. Orvos, D. J. C. Constable, R. E. Hannah, J. Chromatogr., B, **699**, 85 (1995).

4. H. Drossman, J. A. Luckey, A. J. Kostichka, J. D'Cunha, L. M. Smith, Anal. Chem., **62**, 900 (1990).

5. V. Dombek, Z. Stransky, Anal. Chim. Acta, **256**, 69 (1992).

6. T. Higashijima, T. Fuchigami, T. Imasaka, N. Ishibashi, Anal. Chem., **64**, 711 (1992).

7. R. C. Simpson, P. R. Brown, J. Chromatogr., **385**, 41 (1987).

8. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, 2nd edition, Wiley-Interscience, New York, 1979, pp. 51-56.

9. J. J. Kirkland, J. L. Glajch, R. D. Farlee, Anal. Chem., **61**, 2 (1988).

10. J. L. Glajch, J. J. Kirkland, U.S. Patent 4,705,725 (1987).

11. J. J. Kirkland, C. H. Dilks, Jr., J. E. Henderson, LC-GC, **11**, 249 (1993).

12. B. E. Boyes, J. J. Kirkland, Peptide Res., **6**, 249 (1993).

13. J. J. Kirkland, J. W. Henderson, J. Chrom. Sci., **32**, 473 (1994).

14. J. P. Foley, J. G. Dorsey, Anal. Chem., **55**, 730 (1983).

15. M. Verzele, LC, Liq. Chromatogr. HPLC Mag., **1**, 217.

16. Vespalec, J. J. Neca, J. Chromatogr., **281**, 35 (1983).

17. J. Fassberg, V. J. Stella, J. Pharm. Sci., **81**, 676 (1992).

18. J. S Kiel, S. L Morgan, R. K. Abramson, J. Chromatogr., **320**, 313 (1985).

19. J. P. Foley, J. G. Dorsey, Chromatographia, **18**, 503 (1984).

20. "Guidelines for Data Acquisition and Data Quality Evaluation in Environmental Chemistry," Anal.Chem., **52**, 2242 (1980).

Received August 9, 1995
Accepted September 10, 1995
Manuscript 3948

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 625–643 (1996)

COMPARISON OF EXTRACTION METHODS AND COLUMN TYPES FOR THE DETERMINATION OF ADDITIVES BY LIQUID CHROMATOGRAPHY

B. H. Chen,* S. C. Fu

Department of Nutrition and Food Sciences
Fu Jen Catholic University
Taipei, Taiwan 24205, R.O.C.

ABSTRACT

Several extraction methods and column types were compared, with respect to extraction and separation efficiency of 14 additives from soy sauce, sugared fruit and dried roast beef, by paired-ion liquid chromatography. Results showed that the application of a Sep-Pak C_{18} cartridge was the best method for extraction, because it resulted in higher recovery than those given by acetone extraction and steam distillation. The purification of acetone extract by a Sep-Pak silica gel cartridge can result in recovery loss. Monomeric column was superior to polymeric column for simultaneous separation of preservatives, antioxidants and sweeteners. The capacity factor (k') of each additive was also determined.

INTRODUCTION

Food additives, such as preservatives (sorbic acid, sodium dehydroacetate, benzoic acid and p-hydroxybenzoic acid esters), sweeteners (dulcin, saccharin -

Na and acesulfame-K) and antioxidants (BHA and TBHQ) are frequently used in Taiwan to enhance product quality and shelf life. However, it has been reported that the consumption of these additives in excess may exhibit toxic effects to the human body.[1,2,3] Therefore, a method for rapid extraction and simultaneous determination of these additives is necessary.

The extraction of additives from foods has been previously achieved by direct injection,[4,5] steam distillation[6,7] and solvent extraction.[8,9] Direct injection was used for extraction of additives from liquid food samples such as carbonated drinks. This method is simple and easy to use. However, some impurities can also be extracted which interfere with the subsequent separation of additives from foods. Steam distillation can be used for solid and liquid types of foods. However, this method is time-consuming and the recoveries for some additives are low. Organic solvents such as methanol, acetonitrile, ethyl acetate and ethanol can also be used for extraction of additives. However, it is difficult for one solvent to extract all the additives because of polarity differences among these additives. Thus, it is necessary to find a better solvent for the possibility of simultaneous extraction all of these additives. More recently, a Sep-Pak C_{18} cartridge was used for extraction of preservatives and sweeteners in foods.[7] Compared to the other extraction methods, this method is fast and accurate. Also, the recovery is high (>93.8%). Nevertheless, this method has to be modified because some more additives were included in this study. In addition, the possibility of using a Sep Pak™ silica gel cartridge to purify additives has to be investigated.

The simultaneous separation of preservatives, sweeteners and antioxidants by HPLC has been difficult because of polarity differences among these additives. In recent years, paired-ion liquid chromatography was developed for separation of these additives. Terada and Sakabe[7] used a mobile phase of acetonitrile-water-0.2M phosphate buffer solution (7:12:1, v/v/v) containing 2 mM cetyltetrabutylammonium chloride (CTA) to separate nine preservatives and one sweetener, within 32 min, with flow rate at 1.0 mL/min and detection at 233 nm. In a later study, Ikai et al.[9] developed a solvent system of methanol-acetonitrile-0.05M acetonic acid solution (pH = 4.5; 1.5 : 1 : 3.1, v/v/v) containing cetyltrimethylammonium chloride (2.5mM) to separate eight preservatives and one sweetener, within 20 min, with flow rate at 1.0 mL/min and detection at 233 nm. Recently, Chen and Fu[10] employed a mobile phase of acetonitrile-50 mM aqueous α-hydroxyisobutyric acid solution (pH = 4.5; 2.2 : 3.4, v/v) to separate twelve preservatives and three sweeteners, within 40 min, with flow rate at 1.0 mL/min and detection at 233 nm. As these authors used a

monomeric C_{18} phase column to separate additives, it is necessary to compare the separation efficiency between monomeric and polymeric phases of C_{18} columns.

It has been reported that polymeric C_{18} phase exhibits better selectivity for structurally similar compounds than monomeric C_{18} phase.[11] The purposes of this study were (1) to compare the extraction efficiency of four extraction methods, i.e., steam distillation, acetone extraction, Sep-Pak C_{18} cartridge and Sep-Pak silica gel cartridge, and (2) to compare the separation efficiency of additives by monomeric and polymeric phases of C_{18} columns.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a Jasco PU-980 pump, a Jasco UV-970/975 detector (Jasco Co., Tokyo, Japan) and a SIC chromatocorder 12 integrator (System Instruments, Tokyo, Japan). Separations were performed on a Shoko stainless-steel monomeric C_{18} column (25 cm X 4.6 mm I.D., 5μm) (Kyoto, Japan) and a stainless-steel polymeric C_{18} column (25 cm X 4.6 mm I.D., 5μm) (J.T. Baker Co., Frankfurt, Germany). A Sep-Pak C_{18} cartridge, containing 360 mg packing material, and a Sep-Pak silica gel cartridge, containing 690 mg packing material, were all from Waters Co. (Milford, MA, USA)

Reagents

p-Hydroxybenzoic acid esters (methyl-, ethyl-, propyl-, and n-butyl-PHBA), salicyclic acid, 3-t-butyl-4-hydroxyanisole (BHA), and hexadecyltrimethylammonium bromide (HTA) were purchased from Nacalai Co., (Kyoto, Japan). Isopropyl-PHBA, isobutyl-PHBA, and t-butyl hydroxyquinone (TBHQ) were from Tokyo Chemical Co. (Tokyo, Japan). Dulcin, saccharin-Na and α-hydroxyisobutyric acid were from Sigma Co (St.Louis, Mo, USA). Acesulfame-K was from Hoechst Co. (Frankfurt, Germany). Succinic acid and sodium dehydroacetate (DHA-Na) were from Kwok Wah Co. (Taipei, Taiwan). Sodium hydroxide was from J. T. Baker Co. (NJ, USA).

Solvents used for extraction, including methanol, acetonitrile, hexane, ethyl acetate and acetone, were analytical grade and were from Merck (Darmstadt, Germany). HPLC-grade solvents such as methanol and acetonitrile were filtered through a 0.2 μm membrane filter and degassed under vacuum prior to HPLC analysis. All the water used was purified by the Milli-Q water purification system (Millipore, Bedford, MA, USA).

Extraction of Additives by Steam Distillation

A method similar to that used by Terada and Sakabe,[7] for extraction of additives from solid type of foods, was used in our work. A ten gram sample each of soy sauce, roast beef and sugared fruit, was placed in a 500 mL flask. Internal standard TBHQ (250 mg), sodium chloride (60 g), deionized water (150 mL) and 15% tartaric acid solution (10 mL) were added to the flask. The mixture was then steam distilled at a rate of 10 mL/min until approximately 300 mL of distillate was collected in a 500 mL flask; the volume was adjusted to 500 mL with acetonitrile. The solution was filtered through a 0.2 μm membrane filter and subjected to HPLC analysis.

Extraction of Additives by a Sep-Pak C_{18} Cartridge

A modified method used for extraction of additives from both solid and liquid types of foods by a Sep-Pak C_{18} cartridge, as described by Chen and Fu,[10] was used.

Extraction of Additives by Acetone

A two gram sample of soy sauce, and 1 gm sample of sugared fruit and roast beef, each, was placed in a 50 mL volumetric flask. Ten gm sodium chloride was added to the flask, and the volume adjusted with acetone. After swirling vigorously, the mixture was allowed to stand for 30 min, then vacuum-filtered through a Buchner funnel. The filtrate was collected in a 50 mL volumetric flask, and the residue washed with 10 mL acetone. The filtrate was also added to the flask, and the volume adjusted with acetone. The solution was filtered through a 0.2 μm membrane filter and subjected to HPLC analysis.

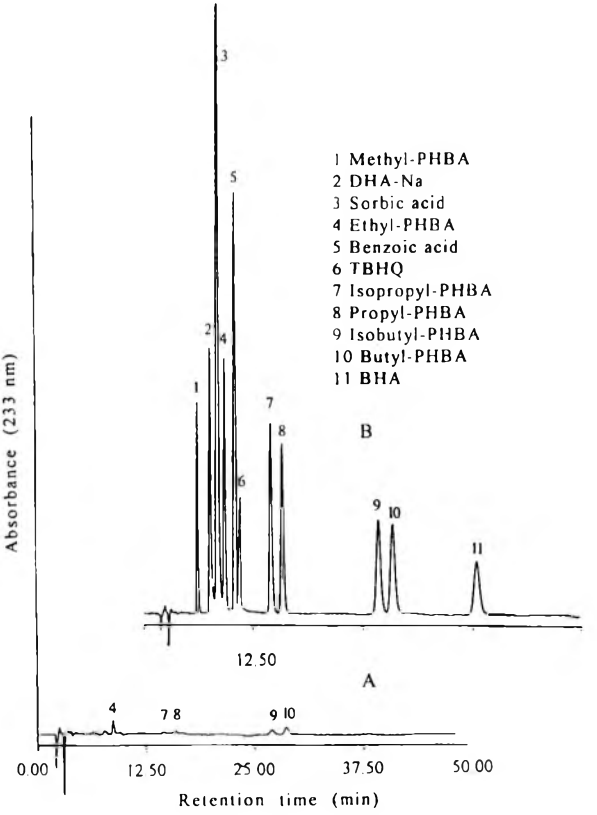


Figure 1. Chromatograms of food additives in soy sauce, extracted by steam distillation. A solvent system consisting of acetonitrile/50 mM aqueous αhydroxyisobutyric acid (pH4.5; 2.2:3.4, v/v) containing 2.5 mM HTA was used. A: soy sauce only; B: soy sauce spiked with food additive standard at a concentration of 25 μg/g each.

Purification of Acetone Extract by a Sep-Pak Silica Gel Cartridge

Five mL acetone extract obtained from 1 gm sample of roast beef, as described above, was poured into a silica-gel cartridge, which was previously activated with 10 mL acetone at a rate of 2.0 mL/min, and filtrate A was collected. Preservatives adsorbing to the packing were then eluted with 10mL ethyl acetate, acetone or methanol; filtrates B, C and D were collected.

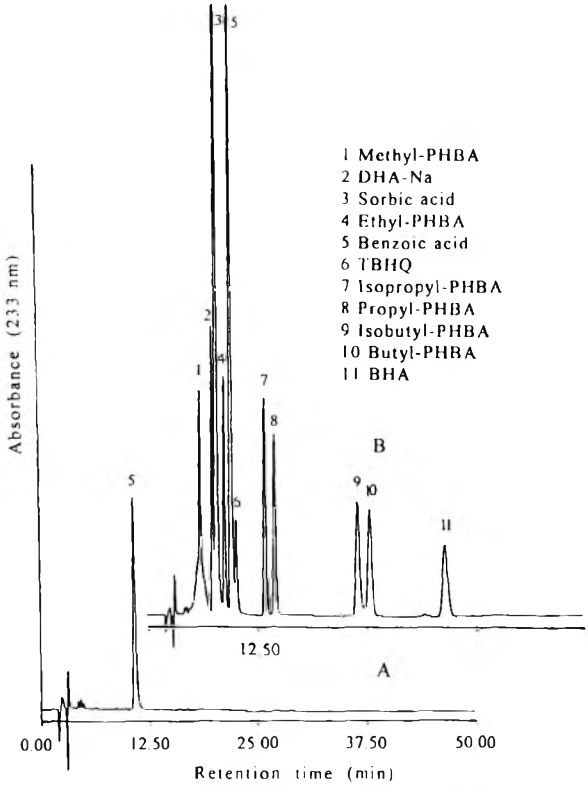


Figure 2. Chromatograms of food additive in sugared fruit, extracted by steam distillation. A solvent system of acetonitrile/50 mM aqueous α-hydroxy isobutyric acid (pH4.5; 2.2/3.4, v/v), containing 2.5 mM HTA was used.
A: sugared fruit only; B: sugared fruit spiked with food additive standard at a concentration of 25 μg/g each.

Each filtrate was diluted to volume (25 mL) with acetone, and filtered through a 0.2 μm membrane filter and subjected to HPLC analysis.

HPLC Analysis of Additives

A mobile phase of acetonitrile-50mM α-hydroxyisobutyric acid solution (pH=4.5; 2.2:3.4, v/v), containing 2.5 mM HTA, was used to separate 14 food

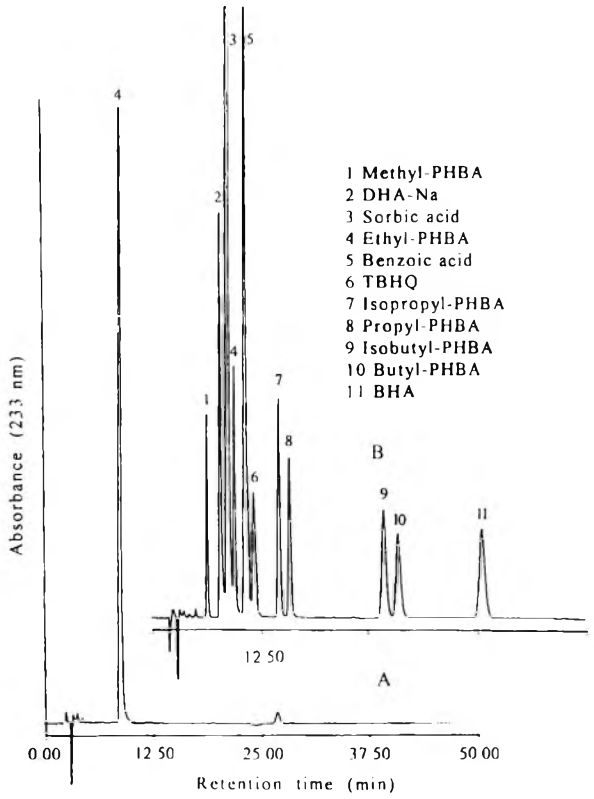


Figure 3. Chromatograms of food additive in dried roast beef extracted by steam distillation. A solvent system of acetonitrile/50 mM aqueous α-hydroxy-isobutyric acid (pH4.5; 2.2/3.4, v/v) containing 2.5 mM HTA was used. A: dried roast beef only; B: dried roast beef spiked with food additive standard at a concentration of 25 μg/g each.

additives, including dulcin, methyl-PHBA, DHA-Na, sorbic acid, ethyl-PHBA, benzoic acid, TBHQ, isopropyl-PHBA, propyl-PHBA, saccharin-Na, acesulfame-K, isobutyl-PHBA, butyl-PHBA and BHA, with detection at 233 nm and flow rate at 1.0 mL/min.[10] Sensitivity was 0.08 AUFS and injection volume was 20 μL. Recovery was determined by adding 25 or 2.5 mg standard to each sample, and steam distillation was performed for the former and the other three extraction methods for the latter. Recovery data were then calculated by dividing the amount of each additive standard added to the sample by the amount of standard obtained following extraction and

Table 1

Recoveries of Food Additives from Various Foods Extracted by Steam Distillation[1,2]

Compound	Recovery[3] (%) Soy Sauce		Sugared Fruit		Dried Roast Beef	
Dulcin	[4]		[4]		[4]	
Methyl-PHBA	86.80[a]	(1.10)	87.39[a]	(0.48)	48.32[b]	(1.35)
DHA-Na	71.63[a]	(2.59)	66.19[b]	(1 20)	65.27[b]	(2.67)
Sorbic Acid	94.77[a]	(3.35)	104.21[b]	(1.22)	52.18[c]	(1.99)
Ethyl-PHBA	80.02[a]	(1.02)	81.46[a]	(2.59)	34.31[b]	(1.81)
Benzoic acid	94.50[a]	(0.56)	109.28[b]	(4.58)	77.00[c]	(2.25)
TBHQ	69.29[a]	(1.84)	83.03[b]	(2.30)	71.35[c]	(1.76)
Isopropyl-PHBA	94.57[a]	(1.65)	97.59[b]	(1.68)	49.64[c]	(1.84)
Propyl-PHBA	90.73[a]	(1.86)	85.95[b]	(3.90)	34.71[c]	(3.44)
Saccharin	[4]		[4]		[4]	
Acesulfame-K	[4]		[4]		[4]	
Isobutyl-PHBA	84.88[a]	(1.12)	83.33[a]	(2.77)	40.38[b]	(1.72)
Butyl-PHBA	85.88[a]	(2.41)	93.05[b]	(2.33)	41.71[c]	(3.44)
BHA	78.42[a]	(5.66)	90.17[b]	(3.51)	65.24[c]	(2.25)

1. a-c symbols bearing different letters in the same row are significantly different ($p<0.05$).
2. Values in parentheses represent coefficient of variation (%).
3. Mean of duplicate determinations.
4. Not extracted by steam distillation.

quantification. Quantitation was carried out using calibration graphs obtained from a standard solution containing six concentrations of additives (5, 10, 25, 50, 75 and 100 ppm) and 25 ppm internal standard (TBHQ).

All the data were subjected to analysis of variance (PROC ANOVA) and Duncan's multiple range test procedures for statistical analysis.[12]

Table 2

Recoveries of Food Additives from Various Foods Extracted by Acetone[1,2]

Compound	Recovery[3] (%) Soy Sauce		Sugared Fruit		Dried Roast Beef	
Dulcin	83.88[a]	(3.13)	78.77[a]	(1.53)	76.42[a]	(1.72)
Methyl-PHBA	87.56[a]	(1.92)	85.00[a]	(1.39)	81.59[a]	(0.97)
DHA-Na	75.04[a]	(2.02)	74.25[a]	(2.39)	69.54[b]	(3.33)
Sorbic Acid	88.39[a]	(1.30)	84.41[a]	(3.66)	89.05[a]	(4.66)
Ethyl-PHBA	82.96[a]	(1.97)	86.04[a]	(2.22)	80.00[a]	(2.49)
Benzoic acid	89.19[a]	(2.27)	89.35[a]	(2.29)	81.54[b]	(1.63)
TBHQ	90.42[a]	(1.87)	88.45[a]	(1.23)	83.51[b]	(1.16)
Isopropyl-PHBA	93.68[a]	(2.78)	91.55[a]	(4.29)	86.74[b]	(1.70)
Propyl-PHBA	92.94[a]	(1.79)	91.76[a]	(1.96)	86.41[a]	(3.88)
Saccharin	89.88[ab]	(2.74)	95.68[a]	(2.25)	84.19[b]	(4.24)
Acesulfame-K	92.76[a]	(1.31)	92.11[a]	(1.89)	85.02[b]	(5.05)
Isobutyl-PHBA	85.44[b]	(4.10)	81.30[b]	(2.21)	85.00[b]	(3.25)
Butyl-PHBA	96.19[a]	(3.25)	92.28[a]	(2.40)	92.76[a]	(1.49)
BHA	88.13[a]	(5.64)	82.88[b]	(3.04)	83.13[ab]	(3.28)

1. a-b symbols bearing different letters in the same row are significantly different ($p<0.05$).
2. Values in parentheses represent coefficient of variation (%).
3. Mean of duplicate determinations.

RESULTS AND DISCUSSION

Extraction by Steam Distillation

Figures 1, 2 and 3 show the HPLC chromatograms of additives in soy sauce, sugared fruit and dried roast beef, extracted by steam distillation, respectively. Five additives, ethyl-, isopropyl-, propyl, isobutyl- and butyl-PHBA were found in the soy sauce, while benzoic acid and sorbic acid were found in both sugared fruit and dried roast beef. Table 1 shows the recoveries

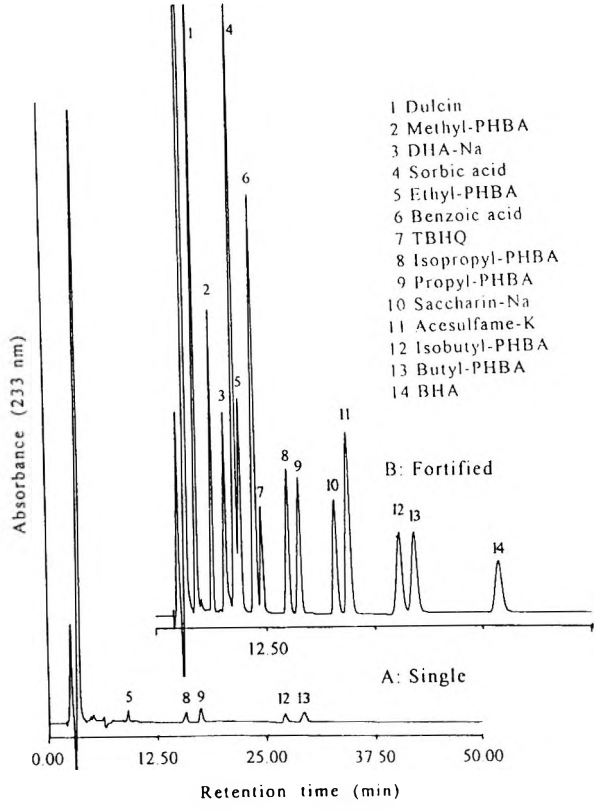


Figure 4. Chromatograms of food additive in soy sauce extracted by acetone. A solvent system of acetonitrile/50 mM aqueous α-hydroxyisobutyric acid (pH4.5; 2.2/3.4, v/v) containing 2.5 mM HTA was used. A: soy sauce only; B: soy sauce spiked with food additive standard at a concentration of 2.5 μg/g each.

of food additives from soy sauces, sugared fruit and dried roast beef by steam distillation. One drawback for steam distillation is that it failed to extract dulcin, saccharin-Na and acesulfame-K from foods. This is probably because sweeteners can be decomposed in the presence of tartaric acid during sample preparation. Compared to the other additives, DHA-Na has lower recovery for soy sauce and sugared fruit, probably because of its high boiling point, which results in partial extraction from food samples. Dried roast beef has lower recovery for all the additives than soy sauce or sugared fruit, mainly because

Table 3

Influence of Various Elution Solvents on Recoveries of Food Additives of Dried Roast Beef Acetone Extract using a Sep-Pak Silica Gel Cartridge

Recoveries[1] (%)

Compound	Acetone Extact	Elution solvent[2] None[3]	Ethyl Acetate[4]	Methanol[4]	Acetone[4]
Dulcin	76.42	61.38	76.99	75.31	75.94
Methyl-PHBA	81.59	81.49	81.52	80.46	82.86
DHA-Na	69.54	44.14	41.91	63.69	51.27
Sorbic Acid	89.05	64.99	75.69	77.46	80.73
Ethyl-PHBA	80.00	80.25	80.46	78.89	81.81
Benzoic acid	81.54	71.54	60.96	62.29	61.63
TBHQ	83.51	83.41	75.28	77.33	76.63
Isopropyl-PHBA	86.74	86.53	79.35	77.81	80.69
Propyl-PHBA	86.41	84.21	80.19	78.46	81.08
Saccharin-Na	84.19	36.92	64.40	71.87	70.22
Acesulfame-K	85.02	69.01	71.72	81.35	71.63
Isobutyl-PHBA	95.00	83.24	85.36	83.19	85.22
Butyl-PHBA	92.76	87.15	90.08	92.56	88.51
BHA	83.13	74.97	78.08	76.46	73.44

1. Mean of duplicate analyses.
2. For purification of acetone extract using a Sep-Pak silica gel cartridge
3. Acetone extract passed through Sep-Pak silica gel cartridge without elution solvent.
4. Acetone extract passed through Sep-Pak silica gel cartridge followed by elution solvent.

the former contains more unwanted substances, i.e., fat and protein, which can interfere with the subsequent separation of additives.

To remedy this problem, the removal of fat and protein is necessary prior to extraction of additives from high fat- or protein-containing foods. Solvents, such as ether, are often employed to extract the fat, followed by potassium

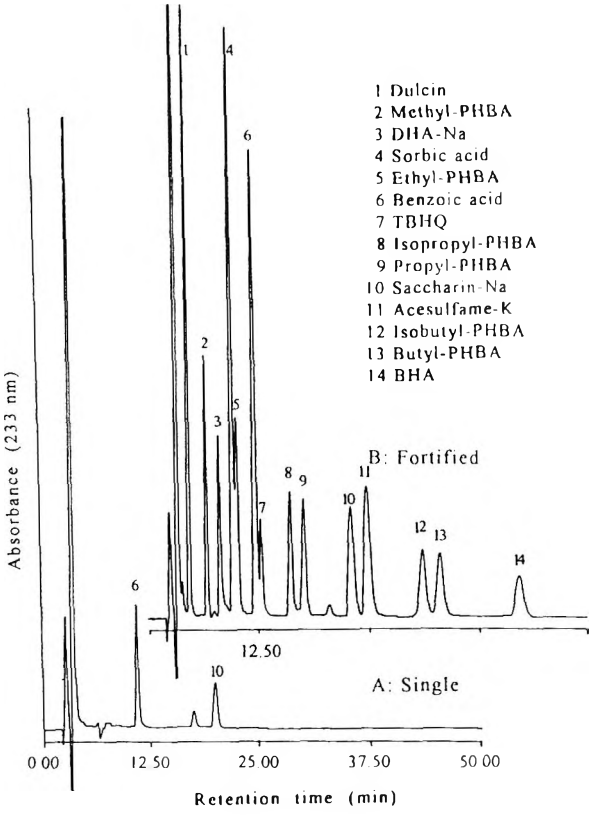


Figure 5. Chromatograms of food additive in sugared fruit extracted by acetone. A solvent system of acetonitrile/50 mM aqueous α-hydroxyisobutyric acid (pH4.5; 2.2/3.4, v/v) containing 2.5 mM HTA was used.
A: sugared fruit only; B: sugared fruit spiked with food additive standard at a concentration of 2.5 μg/g each.

hydroxide, to saponify triglycerides.[13] Likewise, ethanol is often used to precipitate protein.[5]

Nevertheless, the coefficient of variation for all the additives was between 0.48-5.66%, indicating that steam distillation can be applicable to low fat- and protein-containing foods such as soy sauce and sugared fruit.

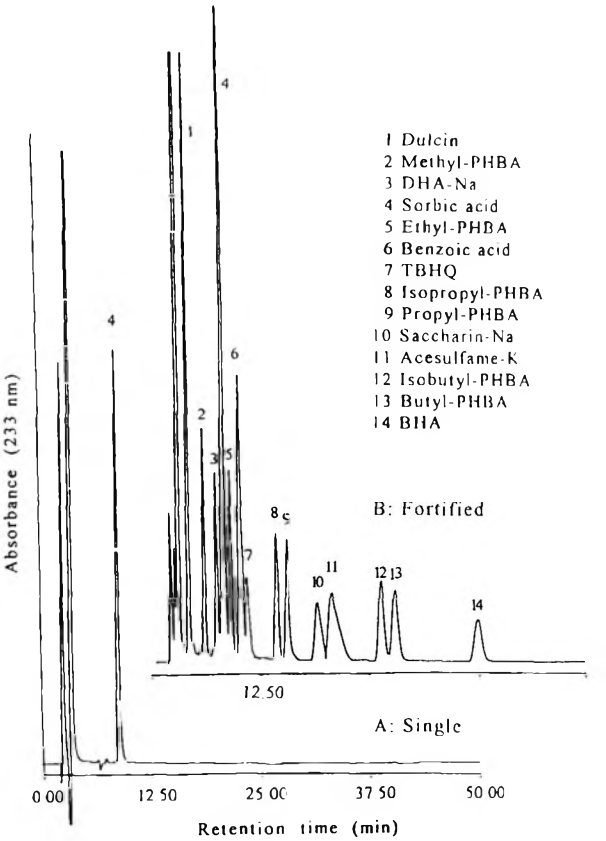


Figure 6. Chromatograms of food additive in dried roast beef extracted by acetone. A solvent system of acetonitrile/50 mM aqueous acetonic acid (pH4.5; 2.2/3.4, v/v) containing 2.5 mM HTA was used.
A: dried roast beef only; B: dried roast beef spiked with food additive standard at a concentration of 2.5 μg/g each.

Extraction by a Sep-Pak C_{18} Cartridge

The recovery data for additives extracted by a Sep-Pak C_{18} cartridge was described in a previous report.[10] Compared to the other additives, saccharin-Na has lowest recovery in dried roast beef, probably because of its partial decomposition under acidic conditions. Unlike steam distillation, the short

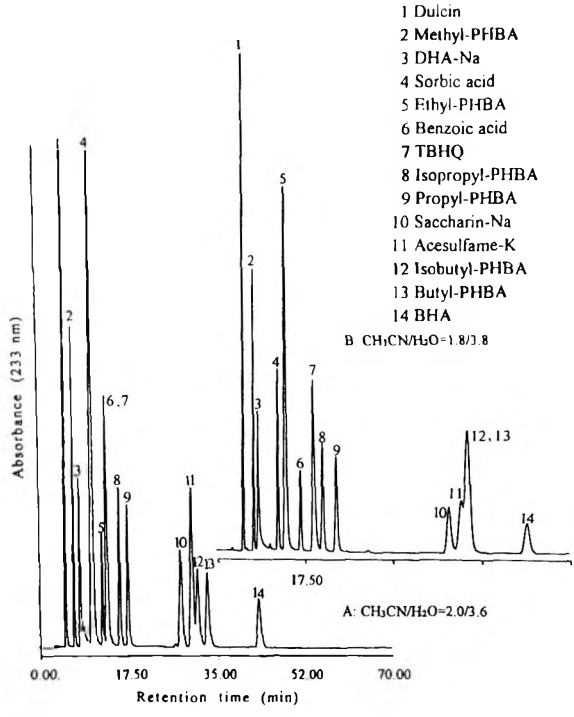


Figure 7. Chromatograms of food additive using a polymeric column and two mobile phases. A: CH_3CN:H_20 = 2.0:3 6; B: CH_3CN:H_2O = 1.8:3.8, containing 50 mM aqueous α-hydroxyisobutyric acid (pH 4.5) and 2.5 mM HTA with detection at 233 nm.

exposure time of saccharin-Na to acid only results in partial decomposition. Also, this method is superior to steam distillation because the latter failed to extract sweeteners from food samples. Nevertheless, one drawback of using a Sep-Pak C_{18} cartridge is that some impurities were also coeluted. Terada and Sakabe[7] used a Sep-Pak C_{18} cartridge to extract additives from coffee drinks and good recovery was obtained.

Our study showed that Sep-Pak C_{18} cartridge can also be applicable to solid type of foods as long as appropriate steps were taken before extraction, and the recoveries of most additives between soild and liquid types of foods were not significant ($p>0.05$).[10]

Extraction by Acetone

It has been reported that organic solvents such as alcohol and ethyl acetate can be used to extract sweeteners and preservatives from foods, and the former (alcohol) results in higher recovery than the latter (ethyl acetate).[14] Figures 4-6 show the HPLC chromatograms of additives in soy sauce, sugared fruit and dried roast beef extracted by acetone, respectively. Soy sauce was found to contain ethyl-PHBA, isopropyl-PHBA, propyl-PHBA, isobutyl-PHBA and butyl-PHBA, while benzoic acid and saccharin-Na were found in sugared fruit and sorbic acid in dried roast beef. Table 2 shows recoveries of additives extracted by acetone. Compared to the other additives, dulcin and DHA-Na have lowest recovery, probably because of their low solubility in acetone. Dried roast beef was also found to have lower recovery for most additives than soy sauce or sugared fruit, mainly because the former contains more protein and fat. Nevertheless, the coefficient of variation was between 0.97 and 5.64%, indicating that using acetone to extract additives can result in high reproducibility.

Purification of Acetone Extract by Sep-Pak Silica Gel Cartridge

From Figures 4-6 it can be seen that dried roast beef contained more impurities than soy sauce and sugared fruit. Thus, it is necessary to investigate the possibility of purifying the acetone extract by employing a Sep-Pak silica gel cartridge, so that column lifetime can be enhanced.

Table 3 shows the effect of various elution solvents on recoveries of additives of dried roast beef using Sep-Pak silica gel cartridge. Without elution solvent, the recovery loss of PHBA esters was low, mainly because of their low polarity, which results in weak interaction with silica gel. In contrast, saccharin-Na has highest recovery loss because of its strong interaction with silica gel. After elution with various solvents, the recoveries of most additives increased. However, saccharin-Na and DHA-Na were only partially eluted, indicating that the solvent strength of elution solvent was too low.

To remedy this problem, the selection of an eluant with high solvent strength, such as water, is necessary. Nevertheless, some impurities can also be coeluted. Thus, using a Sep-Pak silica gel cartridge to purify additives is not an appropriate method.

Table 4

Effect of Monomeric and Polymeric Columns on Capacity Factors (k') of Food Additives[a]

Compound	k' Monomeric C_{18} column	k' Polymeric C_{18} column
Dulcin	0.75	0.65
Methyl-PHBA	1.51	1.18
DHA-Na	2.07	1.61
Sorbic Acid	2.32	2.13
Ethyl-PHBA	2.78	2.13
Benzoic acid	3.15	2.83
TBHQ	3.50	2.83
Isopropyl-PHBA	4.89	3.75
Propyl-PHBA	5.43	4.18
Saccharin-Na	7.24	6.25
Acesulfame-K	7.95	7.00
Isobutyl-PHBA	10.03	7.72
Butyl-PHBA	10.71	8.22
BHA	14.76	11.29

a. A mobile phase of acetonitrile -50 mM aqueous α-hydroxy- isobutyric acid (pH 4.5; 2.2:3.4, v/v), containing 2.5 mM HTA with flow rate at 1.0 mL/min and detection at 233 nm was used.

Comparison of Four Extraction Methods

One drawback of steam distillation is that it failed to extract sweeteners from foods. Despite the drawback, steam distillation can be applicable to extraction of preservatives and antioxidants from liquid, solid or viscous types of foods. Also, the extracts contained less impurities in comparison with the other methods. Sep-Pak C_{18} cartridge is also a good method to choose, because it has higher recovery than the other three methods. The extraction of food additives by acetone is rapid and convenient. However, the acetone extract contained more impurities than the other methods. Also, this method resulted in low recovery for dulcin and DHA-Na in sugared fruit and dried roast beef.

The selection of an appropriate elution solvent is difficult for Sep-Pak silica gel cartridge because of wide polarity difference of additives. Hence, the application of Sep-Pak silica gel cartridge for purification of acetone extract should be disregarded.

Comparison of Monomeric and Polymeric Column

Reverse phase materials can be divided into monomeric and polymeric phases, depending on the difference of modification method on the silica gel surface. The former results from the reaction of monofunctional silane reagent, e.g., chlorodimethyloctylsilane, with silanol sites at the silica surface. Trifunctional silane reagents, e.g., octadecyltrichlorosilane, may also be used to produce monomeric phases. However, silane hydrolysis and polymerization can possibly form a polymeric bonded phase in the presence of water.[11,15] Because of steric hindrance, the selectivity of residual silanol is low and thus solute stability is greatly enhanced.[11]

Table 4 shows the effect of monomeric and polymeric columns on k' (capacity factor) of food additives. Monomeric column is superior to polymeric column in terms of separation efficiency. It has been reported that a polymeric column provided better selectivity for structurally similar compounds.[11] In this study this effect cannot be accounted for, because of wide structural differences among sweeteners, antioxidants and preservatives. Nevertheless, the separation time of using a monomeric column was longer, as shown by k' values. Although the k' values of 14 additives could be reduced to 11.29 by employing a polymeric column, some peaks (sorbic acid and ethyl-PHBA; benzoic acid and TBHQ) were overlapped. This result implied that the solvent strength of the mobile phase was too high. By changing mobile phase as acetonitrile-aqueous α-hydroxyisobutyric acid (2.0:3.6 or 1.8:3.8, v/v) and thus decreasing solvent strength, it was found that the retention times of 14 additives increased substantially (Figures 7A and 7B). Although the separation efficiency was improved, additives benzoic acid and TBHQ coeluted for the former system, and isobutyl-PHBA and butyl-PHBA coeluted for the latter system. From the above discussion, it can be concluded that a monomeric column provided better resolution of 14 additives than a polymeric column as long as a mobile phase of acetonitrile-aqueous α-hydroxyisobutyric acid was employed.

In conclusion, the application of a Sep-Pak C_{18} cartridge is the method of choice because it can extract all the additives from foods and high recoveries

were observed. Monomeric column was found to be superior to polymeric column for simultaneous separation of preservatives. sweeteners and antioxidants.

ACKNOWLEDGMENT

This study was supported by a grant (NSC84-409-B-030-002) from the National Science Council, Taiwan, R.O.C.

REFERENCES

1. J. Y. Hsu, **The Proper Use of Additive**, Hua-Siang-Yuan Publishing Co., Taipei, 1985, pp.227-333.

2. J. J. Chen, G. R. Shen, **Food Additive**, Wen-Yuan Publishing Co., Taipei, 1986, pp.173-201.

3. H. W. Chiu, Food Industry, **18**, 36-38 (1986).

4. M. Veerabhadrarao, M. S. Narayan, O. Kapur, J. Assoc. Off. Anal. Chem., **70**, 578-582 (1987).

5. J. F. Lawrence, C. F. Charbonneau, J. Assoc. Off Anal. Chem., **71**, 934-937 (1988).

6. AOAC, Assoc. Offic. Anal. Chem., 14th ed. Washington, D.C., 1984.

7. H. Terada, Y. Sakabe, J. Chromatogr., **346**, 333-340 (1985).

8. L. V. Bui., C. Cooper, J. Assoc. Off Anal. Chem., **70**, 892-896 (1987).

9. Y. Ikai, H. Oka, N. Kawamura, M. Yamada, J. Chromatogr., **457**, 333-343 (1988).

10. B. H. Chen, S. C. Fu, Chromatographia, In Press, 1995.

11. L. C. Sander, S. A. Wise, Anal. Chem., **56**, 504-510 (1984).

12. **SAS/STAT Guide for Personal Computer**, Version 6 ed, SAS Instruments, Cary, NC, 1985.

13. U. Leuenberger, R. Gauch, E. Baumgartner, J. Chromatogr., **173**, 343-348 (1979).

14. K. H. Wu, H. F. Lee, M. H. Shen, Annual Report of Food and Drug Bureau of Taiwan, **1**, 246-249 (1989).

15. M. Verzele, P. Mussche, J Chromatogr., **254**, 117-122 (1983).

Received August 13, 1995
Accepted September 5, 1995
Manuscript 3949

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 645–659 (1996)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ALACHLOR IN ALGINATE-BASED FORMULATIONS

J.W. Kuan, A.B. Pepperman

Southern Regional Research Center
1100 Robert E. Lee Boulevard
New Orleans, Louisiana 70124

ABSTRACT

A reverse phase HPLC method for the quantitative determinations of alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide] and its degradation products in alginate-based alachlor controlled release formulations has been developed. Sonication and the addition of sodium ethylenediaminetetraacetate as a chelating agent were employed to disintegrate the formulation matrix. The disintegrated formulations were extracted with acetonitrile. The acetonitrile extracts were analyzed for alachlor and its degradation products by HPLC. A C_{18} column with a mobile phase of 65% acetonitrile and 35% water was used for separation. A UV detector set at 215 nm was selected for quantitation, and a photodiode array detector was used for confirmation. The developed method was used for the determinations of the percent active ingredient in freshly prepared and aged alachlor formulations made with and without oil (linseed, soybean, or corn oil). The method was also applied to monitor the rate of release and the fate of alachlor in controlled-release studies of alachlor formulations in water.

Alachlor degradation products, N-demethoxymethylalachlor and 2,6-diethylaniline (trace), were found in the formulations with oil but not in formulations without oil. The degradation of alachlor continued slowly as the formulations aged.

INTRODUCTION

Alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide] is a preemergence herbicide used for weed control in agronomic corps such as corn, soybean, peanut, rice, and potato. It acts as a herbicide by inhibiting protein synthesis and root elongation in susceptible plants.[1] Alachlor is one of the most widely used herbicides in the United States, with an annual application rate of about 85 million pounds.[2] Through its regular use, improper disposal, and accidental spills, alachlor was one of the pesticide contaminants most often found in the groundwater and wells.[3-5]

Alachlor is available commercially as an emulsifiable concentrate, clay granules, and microcapsules. The alachlor in commercial formulations is commonly assayed by organic solvent extraction followed by gas chromatography (GC). Typically, solvent or solid phase extraction is used to extract alachlor in water and soil, and the alachlor is quantitatively determined by GC or high performance liquid chromatography (HPLC).[6,7] GC/MS or LC/MS were usually employed for the quantitation, identification, and confirmation of alachlor and its metabolites.[8-10] Recently, due in part to the commercial availability of several alachlor enzyme-linked immunosorbent assay (ELISA) kits, this technique has been accepted and used for the analysis of alachlor in environmental water and food samples.[11,12] Despite drawbacks such as occasional false positives[13] and low cross-reactivity with metabolites, ELISA is still a good tool for the screening of environmental samples because it is rapid, simple to operate, inexpensive, and portable.

Herbicide controlled release formulations (CRFs) have potential benefits of enhanced weed control and corp protection, improved safety of handling, reduced losses to volatilization and leaching.[14] Connick et al.,[15] and Pepperman and Kuan[16] have demonstrated that incorporated clay and oil into herbicide-alginate formulations retards the release of herbicides. We have incorporated clay and oil into alginate-based alachlor controlled release formulations in our continuing search for a CRF which exhibits the best efficacy and has the least adverse impact on the environment.

After curing, our alginate-based formulations that incorporated oil and other adjuvants such as clay formed rather rigid beads which were not readily dissociated. Traditional methods involving simple organic solvent extraction did not recover all of the alachlor in the formulations. In a previous report, an HPLC method for the determination of percent active ingredient (% a.i.) in metribuzin-alginate formulations was described.[16] In this method, the % a.i. was obtained indirectly by the analysis of the formulation filtrates. The method was satisfactory for the determination of % a.i. in freshly prepared formulations, but could not be employed for the residual analysis of herbicide formulations that had been applied to the soil or partially extracted with water. The objective of the present study was to develop a direct method for the analysis of alachlor and its degradation products in the alginate-based formulations to monitor the fate of alachlor in the formulations at various stages of usage and release.

MATERIALS AND METHODS

Chemicals

Alachlor [technical grade, 94% pure] and N-demethoxymethylalachlor [2-chloro-N-(2,6-diethylphenyl)acetamide, 99.7% pure] were supplied by Monsanto Company, St. Louis, MO.[17] Technical grade alachlor was recrystallized from an ethyl acetate-hexanes mixture at 4 °C to a purity of about 99% with a mp of 40.5-41.5 °C. 2,6-Diethylaniline [99.5+% pure] was purchased from Aldrich Chemical Co., St. Louis, MO. Sodium alginate, Kelgin MV, was provided by Kelco, Division of Merck and Company, San Diego, CA, and kaolin clay was supplied by Thiele Kaolin Company, Wren, GA. Tween 20 [polyoxyethylenesorbitan monolaurate] and tetrasodium ethylenediaminetetraacetate dihydrate (sodium EDTA) were purchased from Sigma Chemical Co., St. Louis, MO. Raw linseed oil was purchased from a local hardware store, and soybean oil and corn oil were obtained from a local supermarket. Water purified with a NANOpure Ultrapure Water System (Barnstead/Thermolyne Corp, Dubuque, Iowa) through a 0.2 um final filter was used throughout the study. All other chemicals were either HPLC grade or reagent grade.

Apparatus

All HPLC analyses were performed on a Waters HPLC system (Waters Chromatographic Division, Millipore Corp., Milford, MA.). The system

consisted of a model 712 WISP auto-sampler, a model 600E Powerline multisolvent delivery system, a data system with model 991 Photodiode Array Detector V.6.22A Powerline software, a model 5200 printer/plotter, and two detectors: a model 486 IEEE tunable absorbance detector with analytical flow cell and a model 991 photodiode array detector (PDA). The detectors were installed parallel to each other and an automated switching valve was used to switch the direction of the flow to the detector. The HPLC was fitted with a Waters Nova-Pak C_{18} stainless steel column, 300 mm long x 3.9 mm i.d., 4 micron particle size. The mobile phase was 65% acetonitrile and 35% water at a flow rate of 0.7 mL/min. The solvents were sparged with helium at the flow rate of 30 mL/min. For quantitation, the model 486 detector was selected and was set at 215 nm, 1 AU full scale. Injection volume was 20 ul, each sample was injected twice, and the run time was 14 min. The PDA detector was used to acquire UV spectra and for confirmation of alachlor and the degradation products

Sonication was performed in an ultrasonic cleaner with a tank capacity of 3 L. and the dimensions of 3.75" H x 9.5" L x 5.5" W, model # SC-100H (Ultrasonic Industries, Clearwater, FL).

METHODS

Preparation of Alachlor Formulations

A typical alachlor formulation was prepared by first dissolving alachlor (1%) in methanol (5%). (All percentages herein are by weight, w/w). The oil (0-10%) and Tween 20 (0.5%) were added and the mixture was mixed with an overhead stirrer. While stirring the mixture at 200-250 rpm, the water (72.5-82.5%) was added very slowly to the mixture. Caution was taken not to add water too rapidly at the beginning to prevent precipitation and aggregation of alachlor. The clay (10%) was added, and the mixture was stirred at 350 rpm for 10 min. The sodium alginate (1%) was then added, and the mixture was stirred at 450 rpm for 1 hr. or until a homogeneous slurry was obtained. The slurry was added dropwise through Pasteur pipets into 0.25 M calcium chloride (twice the weight of the slurry) to form calcium alginate gel beads. The beads were weighed and allowed to harden for about 5 min. The liquid was removed by vacuum filtration through a coarse-frit Buchner funnel. The beads were rinsed with water and drained. The wet beads were spread on aluminum foil to air-dry at room temperature. Although the beads were essentially dry in 24 hours, they were dried for two weeks to allow for formation of a polymeric film

on the surface of the beads.[16] The formulation filtrate and its rinsate were combined and saved for the HPLC determination of % a.i. The % a.i. obtained from the analysis of the formulation filtrate was considered an "indirect method" for the determination of % a.i. in the formulation.

The color of the dried beads was off-white for formulations without oil and beige to light yellow-brown for oil-containing formulations. They were all spherically shaped with a diameter ranging from 1.5 mm (0% oil) to 1.7 mm (10% oil).

Preparation of Standards

Stock standards of alachlor, N-demethoxymethylalachlor, and 2,6-diethylaniline, 1.00 mg/mL, were prepared in HPLC grade acetonitrile. Working standard solutions of these compounds, 1-100 ppm, were prepared by diluting the stock standards with mobile phase (65:35 = acetonitrile:water). Working standard solutions were used for the construction of calibration curves. Standard alachlor solution was run routinely as a control.

Sample Preparation for HPLC Analysis

A formulation sample containing less than 10 mg of alachlor, ca. 0.1-0.2 g, was accurately weighed in an 8-dram (25x95 mm) vial with a Teflon-lined screw cap. Four mL of 0.05 g/mL (0.12 M) sodium EDTA was added to the sample. The vial was capped and mixed with a vortex mixer for 30 sec before it was placed in an ultrasonic bath. The sample mixture was sonicated for 10 min, followed by 30-60 sec of mixing with a vortex mixer. The total sonication time was 20-30 min or until the formulation was completely disintegrated. The sample was extracted with 8 mL of acetonitrile, and was mixed with a vortex mixer for 1-2 min. The extract was allowed to stand 10-20 min or until a clearly-defined phase separation occurred and the precipitate settled. The acetonitrile (upper) layer was then transferred to a clean vial and diluted to 15 mL with acetonitrile. A 1.0 mL aliquot of the diluted extract was further diluted with 3 mL of acetonitrile to precipitate more alginate. It was mixed with a vortex mixer for 30 sec then filtered through a 0.22 um Millex-GV filter (Millipore Corp., Bedford, MA). Before injecting into the HPLC, 2 mL of filtrate was mixed with 1.0 mL of water.

The combined formulation filtrate-rinsate was diluted to a known volume

with water. For HPLC analysis, 1.05 mL of diluted filtrate was mixed with 1.95 mL of acetonitrile. The mixture was filtered through a Millex-GV filter before injecting into the HPLC. The extract from the controlled release studies of alachlor formulations was prepared in the same manner as the filtrate.

RESULTS AND DISCUSSION

Factors Affecting the Analysis

1) Disintegration of alginate beads with EDTA

Water soluble sodium alginate was used to form a slurry with the herbicide and the other ingredients. The mixture when dropped into calcium chloride solution formed water insoluble calcium alginate beads. Calcium alginate can be rendered soluble by the addition of a ligand which will displace alginate and produce a water soluble calcium chelate. Sodium salts of citric acid, phosphoric acids such as tripolyphosphate and hexametaphosphate, and EDTA have been the most commonly used chelating agents to sequester the calcium in alginates. The stability constants (formation constants), log K, of these calcium chelates are: citrate 3.5; tripolyphosphate 5.2; hexametaphosphate 6.0; EDTA 10.7.[18] Because alachlor is not very stable in acidic solutions and EDTA has the strongest chelating power (highest formation constant), EDTA was selected as the chelating agent for the disintegation of alachlor-alginate beads. At pH 10-11, almost all the EDTA is non-protonated providing free EDTA ions for chelation with calcium. Sodium EDTA solutions, 0.05 g/mL (0.12 M) and 0.1 g/mL (0.24 M) which had pH of 10.7 and 10.9, respectively, were tested on an alachlor-oil-alginate formulation. The % a.i. obtained from using 0.1 g/mL was about 6% lower than the one with 0.05 g/mL. An EDTA concentration lower than 0.05 g/mL can be used for disintegration but it may require a longer sonication time. If an EDTA concentration higher than 0.1 g/mL (pH>11) is used, sodium alginate, specifically Kelgin MV, can depolymerize due to hydrolysis. The depolymerization produces many water soluble small molecules which may interfere with the alachlor analysis.

2) Sonication time

Sonication was employed to disintegrate the beads. The time required for complete disintegration of the beads depended on the oil content and the age of the beads. Formulations with no oil took less time than formulations with oil.

Also, aged formulations required a longer sonication time than fresh ones. It took about 20 min for the non-oil formulation and 30-80 min for oil formulations, depending on the percent oil used in the formulation and age of formulation, to disintegrate the beads completely. It is recommended that a brief pause be taken after each 10 min of sonication. The samples are removed from the sonication bath and swirled at high speed for 30-60 sec with a vortex mixer. Interrupted sonication prevents sample overheating which results in alachlor decomposition. Swirling of the samples aids in speeding up the disintegration of the beads. Crushing the beads prior to the sonication shortened the sonication time for the complete disintegration of non-oil formulations, but was not effective for the oil-containing formulations. Oil-containing beads, especially those with 8-10% oil, tended to flatten and stack together rather than crumble to small particles when crushed. Although it has been reported[19] that alachlor in water decomposed after lengthy sonication, no significant decomposition was found in our samples due to sonication.

3) Solvent extraction

Acetonitrile and ethyl acetate were evaluated as potential solvents for the extraction of alachlor from the EDTA-formulation mixture. Alachlor is very soluble and readily extractable in either solvent. However, with the use of ethyl acetate, multiple extractions and evaporation of the solvent from the extract prior to analysis were necessary. Since acetonitrile was used in the mobile phase, no drying of the extract was required when acetonitrile was used for sample extraction. Three different acetonitrile extraction procedures were tested on the same formulation: (1) once with 8 mL, (2) twice with 4 mL each, (3) first with 6 mL, second with 4 mL. The acetonitrile extracts were removed, combined (in 2 & 3), and diluted to 15 mL with acetonitrile. Alachlor was determined as described above. The % a.i. obtained for all three extraction procedures were the same. Hence, acetonitrile was chosen as the extraction solvent and each sample was extracted only once with 8 mL of acetonitrile.

Several problems were noted when acetonitrile was investigated as the extraction solvent. When the percentage of acetonitrile in the extraction mixture was lower than 50%, the extract was cloudy and it took a long time for the precipitates to settle. When the percentage was higher than 75%, the extract was very cloudy, and the precipitate became pasty and adhered to the wall of the vial when mixing, therefore, the phase separation was hard to attain and observe. Optimum extraction conditions were obtained when the percentage of acetonitrile in the extraction mixture ranged from 60 to 70%, e.g. acetonitrile:water = 3:2 to 2:1.

4) Absorption of alachlor on Millex-GV filters

During the development of the HPLC method for the analysis of alachlor, some absorption of alachlor on the Millex-GV filters was observed. To quantify the absorption, two experiments were conducted. In the first experiment, two sets of identical alachlor standards, 3 mL each, concentration: 10, 25, 100 ppm, were prepared in the purified water. One set of the standards was filtered through 0.22um Millex-GV filters, and the other set of standards was not filtered before the injections. The alachlor found in the filtered standards was only 77-79% of that found in the non-filtered ones. The percent absorption was similar regardless of the alachlor concentrations for the range tested (10-100 ppm). In the second experiment, standards were prepared directly in the mobile phase (acetonitrile:water = 65:35). No significant absorption of alachlor by the filters was observed.

Recovery Study

To verify the accuracy of the developed HPLC method for the determination of % a.i. in the alachlor formulations, a recovery study was conducted on an alachlor-linseed oil-alginate-clay formulation. The samples, in duplicate, were spiked with 0, 1, 3, 5 mg of alachlor. The average percent recovery of alachlor ranged from 96.5 to 101.3%.

Direct vs. Indirect Method for the Determination of % a.i. in the Formulations

Before developing the current method, designated as "direct method", the % a.i. in the alachlor formulation was determined by the HPLC analysis of alachlor in the formulation filtrate. The calculation of % a.i. in the formulation was based on the assumption that whatever was not found in the filtrate should be in the dried formulation. The % a.i. obtained by this method was considered an "indirect method". It was calculated as follows:

$$\% \text{ a.i.} = (A*W/T - F)*100/D$$

where: A= wt. of alachlor used in the preparation of formulation
W= wt. of wet beads (droppings in calcium chloride)
T= wt. of total ingredients
F= wt. of alachlor found in the filtrate
D= wt. of the dried formulation

Table 1

Direct vs. Indirect Method for the Determination of % Active Ingredient (a.i.) in the Formulations

Formulation	Direct, % a.i.		Indirect % a.i.**	Percent of direct/indirect	
#: oil type	ALC only	ALC+DMA		ALC only	ALC+DMA
A: no oil	6.63	6.63	7.01	94.6	94.6
B: linseed oil	4.67	4.84	5.27	88.6	91.8
C: soybean oil	4.63	4.83	5.22	88.7	92.5
D: corn oil	4.60	4.78	5.26	87.5	90.9

Formulations A,B,C,D all contained 1% alachlor, 10% clay, 1% alginate.
Formulation A= no oil.
Formulations B,C,D= 4% oil.
ALC, alachlor; DMA, N-demethoxymethylalachlor.
*Average of eight determinations.
**Average of four determinations.

A comparison study of these two methods was conducted on four formulations by analyzing both their filtrates (indirect method) and the dried formulations (direct method). These four formulations all had 1% alachlor, 10% clay, and 1% alginate, but A contained no oil and B, C, D contained 4% linseed oil, soybean oil, and corn oil, respectively. The average % a.i. obtained from four determinations by the indirect method and eight determinations by the direct method are shown in Table 1. The % a.i. determined by the direct method was consistently lower than by the indirect method. No significant alachlor decomposition products were found in either the filtrates or in extracts of the non-oil formulation. However, two alachlor degradation products, identified as N-demethoxymethylalachlor (DMA) and 2,6-diethylaniline (DEA) were found in oil-containing formulation extracts.

For the non-oil formulation (A), the % a.i. obtained by the direct method was about 95% of the value obtained by the indirect method, while the ratio of the two methods, direct/indirect, was 87-89% for the formulations containing oil (B,C,D). The relatively lower values of % a.i. by the direct method for formulations containing oil were partially due to the decomposition of alachlor

which was not accounted for. If the concentrations of DMA found in the formulations were included in the calculation, the ratio of direct/indirect was about 91-93%. DEA was not included in the calculations because only trace amounts of it were found. In the indirect method, small losses of alachlor on aluminum foil and to volatilization during the drying/curing stage (usually two weeks to allow linseed oil to cure) could not be accounted for in the calculation of % a.i. Judging from the recovery study and due to the possible losses of a.i. which can occur in the indirect method, the % a.i. obtained from the direct method should more accurately reflect the actual amount of the herbicide present in the beads.

Degradation of Alachlor in the Formulations

The presence of DMA and trace amounts of DEA in the formulations were identified and confirmed by comparison with the authentic compounds run under the same HPLC conditions. The retention time (RT) and response factor (RF) of DMA, DEA, and alachlor (ALC) were determined by the use of standards under the same conditions (see HPLC method). The RT and their relative RT were: DMA:ALC:DEA=5.39:9.40:10.00=0.5734:1.0000:1.0638. The relative response factors (RRFs) were DMA:ALC:DEA= 0.9689:1.0000:0.9575. The concentrations of DMA and DEA were calculated, based on their RRFs.

DMA is a major degradation product of alachlor in soil and water.[20,21] In the formulations we prepared and stored at room temperature, DMA was found only when oil was present and DMA concentration increased as the formulations aged. About 3% of alachlor in the formulations degraded to DMA two weeks after the preparation. The conversion continued slowly to about 5% in two months, and increased to 6-7% in 7 months. Formulations containing linseed oil had slightly slower degradation rates than formulations containing soybean oil or corn oil. A two-months-old formulation with linseed oil converted 4.4% alachlor to DMA, while soybean oil (5.5%) and corn oil (5.7%) formulations were higher in DMA. Since no significant alachlor degradation products were found in filtrates and non-oil formulations but were found in all formulations containing linseed, soybean, and corn oil, it is reasonable to believe that the decomposition of alachlor was caused by the interaction between alachlor and the oil in the formulations. The degradation of alachlor is affected by temperature, moisture, and surrounding environments. DMA was reported as a major degradation product from the hydrolysis of alachlor in acidic medium, and as one of the alachlor metabolites produced by soil

fungi.[22,23] DMA found in the oil-containing formulations probably resulted from the interaction of alachlor with free fatty acids in the oil or with products from the decomposition and/or oxidation of the oil. Free fatty acids have been reported as impurities in alginates from different sources.[24] Since there was no obvious degradation of alachlor in the non-oil formulation, it appeared that possible impurities in the alginate had no significant effect on the degradation. Alginates are hydrophilic polysaccharides which readily absorb moisture from the atmosphere. Linseed oil, being a drying oil, polymerized and formed a hard film on the surface of the beads.[16] The hard film on the beads may act as a barrier to protect the beads from oxidation and moisture, thus slowing the rate of alachlor decomposition. Soybean oil is a semi-drying, and corn oil a non-drying oil, hence, the alachlor decomposition rates were higher in formulations C and D than in B.

Determination of Alachlor and the Degradation Products in Partially Extracted Formulations

Controlled release studies were performed on some of the alachlor formulations in either static water and/or agitated (by shaking) water to determine the herbicide release properties. In the static water test (see ref. 25 for method), the release of alachlor from the formulations usually reached an equilibrium in a few days and no further increase of concentration was observed. To investigate the fate of the unreleased alachlor, an attempt was made to analyze the residual alachlor in those partially extracted formulations. Both original (non-extracted) and partially extracted formulations were analyzed by the developed method reported herein. Besides alachlor, DMA was the only other detectable compound found in the formulations. For partially extracted formulations, the total alachlor concentration was calculated by the addition of alachlor and DMA (converted to alachlor equivalent concentration) found in the water extracts and in the formulations.

The results of the controlled release study (CRS) in static water and the residue analysis of four representative alachlor formulations, in duplicate, are shown in Table 2. In comparison with the original formulations, the average overall percent recovery of alachlor from water extracts and partially extracted formulations were 96.7 to 99.3%. DMA was found in the extracts and the residues of the oil-containing formulations but was not found in non-oil formulation. Overall percent recovery increased slightly from 97.0-99.3% to 97.4-99.7% for oil-containing formulations if DMA was included in the calculations.

Table 2

Determination of Alachlor and Its Degradation Products in Partially Extracted Formulations

Formulation	Orig. Formulation		Amt. in Extract*		Resid. Formulation		Overall % Recovery	
#: Oil Type	Av. % ALC	Av. % ALC+DMA	ALC mg	ALC+DMA mg	% ALC	% ALC+DMA	ALC	ALC+DMA
E: no oil	6.92	6.92	67.66	67.66	4.87	4.87	94.5	94.5
			67.80	67.80	5.20	5.20	98.8	98.8
F: linseed oil	4.67	4.91	43.43	51.37	3.91	3.97	100.0	100.3
			43.94	51.94	3.82	3.88	98.6	99.0
G: soybean oil	4.67	4.97	41.97	52.14	3.86	3.93	97.1	97.6
			48.83	59.49	3.76	3.81	98.8	99.1
H: corn oil	4.70	5.01	46.97	57.94	3.71	3.76	96.4	96.8
			50.37	61.57	3.68	3.73	97.6	98.0

Formulations E,F,G,H all contained 1% alachlor, 10% clay, 1% alginate.
Formulation E= no oil.
Formulations F,G,H= 4% oil.
ALC, alachlor; DMA, N-demethoxymethylalachlor.
*Amount found in the extracts after formulations had been in static water for 96 hrs.

CONCLUSION

The developed reverse phase HPLC method allowed quantitation and identification of alachlor and its degradation products in alginate-based alachlor formulations. The method was also used to obtain the alachlor release profiles and the degradation rates in the controlled release studies of alachlor formulations in water. The major degradation product found in the alachlor-oil-alginate formulations was N-demethoxymethylalachlor. Either trace amounts or none of 2,6-diethylaniline were found in those formulations. The extent of alachlor degradation depended on the age and the oil type in the formulations (linseed< soybean < corn oil). The method as developed can be used to monitor the environmental fate of alginate-clay-oil containing alachlor formulations in the field or greenhouse. Some modification of the extraction method for soil samples may be necessary.

REFERENCES

1. L. M. Deal, F. D. Hess, " An Analysis of the Growth Inhibitory Characteristics of Alachlor and Metolachlor," Weed Science, **28**, 168-175 (1980).

2. **National Research Council. Regulating Pesticides in Food: The Delaney Paradox**, National Academy Press, Washington D.C. (1987) pp.52-53.

3. **Nonpoint Source Pollution in the U.S.**, U.S. Environmental Protection Agency, Report to Congress (1984).

4. S. Z. Cohen, S. M.Creeger, R. F. Carsel, C. G. Enfield, "Potential Pesticide Contamination of Groundwater from Agricultural Uses," in **Treatment and Disposal of Pesticide Wastes**, ACS Symposium Series No. 259, American Chemical Society, Washington D.C. (1984), pp.297-325.

5. L. R. Holden, J. A. Graham, R. W. Whitmore, W. J. Alexander, R. W. Pratt, S. K. Liddle, L. L. Piper, "Results of the National Alachlor Well Water Survey," Environ. Sci. Technol., **26**, 935-943 (1992).

6. J. M. Warner, D. D. Arras, "Alachlor," in **Comprehensive Analytical Profiles of Important Pesticides**, a volume in the series Modern Methods for Pesticide Analysis, J. Sherma, T. Cairns, eds., CRC Press, (1993), pp.113-115.

7. M. Negre, M. Gennari, E. Raimondo, L. Celi, M. Trevisan, E. Capri, "Alachlor Dissipation in Soil as Influenced by Formulation and Soil Moisture," J. Agric. Food Chem., **40**, 1071-1075 (1992).

8. L. Q. Huang, "Simultaneous Determination of Alachlor, Metolachlor, Atrazine, and Simazine in Water and Soil by Isotope Dilution Gas Chromatography/Mass Spectrometry," J. Assoc. Off. Anal. Chem., **72**, 349 (1989).

9. W. E. Pereira, C. E. Rastad, T. J. Leiker, "Determination of Trace Levels of Herbicides and their Degradation Products in Surface and Ground Waters by Gas Chromatography/Ion-Trap Mass Spectrometry," Anal. Chim. Acta, **228**, 68-75 (1990).

10. R. D. Voyksner, J. T. Bursey, E. D. Pellizzari,. "Analysis of Selected Pesticides by High Performance Liquid Chromatography-Mass Spectrometry," J. Chrom., **312**, 221-235 (1984).

11. P. C. C. Feng, S. J. Wratten, S. R. Horton, C. R. Sharp, E. Logusch, "Development of an Enzyme-Linked Immunosorbent Assay for Alachlor and its Application to the Analysis of Environmental Water Samples," J. Agric. Food Chem., **38**, 159-163 (1990).

12. S. J. Lehotay, R. W. Miller, "Evaluation of Commercial Immunoassays for the Detection of Alachlor in Milk, Eggs and Liver," J. Environ. Sci. Health, **B29**, 395-414 (1994).

13. D. B. Baker, R. J. Bushway, S. A. Adams, C. Macomber, "Immunoassay Screens for Alachlor in Rural Wells: False Positives and an Alachlor Soil Metabolite," Environ. Sci. Technol., **27**, 562-564 (1993).

14. B. D. Riggle, D. Penner, "The Use of Controlled-Release Technology for Herbicides," Rev. Weed Sci., **5**, 1-14 (1990).

15. W. J. Connick, Jr., J. W. Bradow, W. Wells, K. K. Steward, T. K. Van, "Preparation and Evaluation of Controlled-Release Formulations of 2,6-Dichlorobenzonitrile," J. Agric. Food Chem., **32**, 1199-1205 (1984).

16. A. B. Pepperman, J. W. Kuan, "Slow Release Formulations of Metribuzin Based on Alginate-Kaolin-Linseed Oil," J. Controlled Release, **26**, 21-30 (1993).

17. This article reports the results of research only. Mention of a pesticide in this article does not constitute a recommendation for use by the U.S. Department of Agriculture nor does it imply registration under FIFRA as amended. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

18. L. G. Sillen, A. E. Martell, eds. **Stability Constants of Metal-ion Complexes. Supplement No.1**, Special Publication No.25, The Chemical Society, London, (1971), pp.118,411,624.

19. W. C. Koskinen, K. E. Sellung, J. M. Baker, B. L. Barber, R. H. Dowdy, "Ultrasonic Decomposition of Atrazine and Alachlor in Water," J. Environ. Sci. Health, **B29**, 581-590 (1994).

20. E. Capri, A. Walker, " Biodegradation of Liquid and Microencapsulated Formulations of Alachlor in a Clay Loam Soil," Bull. Environ. Contam. Toxicol., **50**, 506-513 (1993).

21. R. S. Hargrove, M. G. Merkle , "The Loss of Alachlor from Soil," Weed Sci., **19**, 652-654 (1971).

22. J. M. Tiedje, M. L. Hagedorn, "Degradation of Alachlor by a Soil Fungus, *Chaetomium globosum*," J. Agric. Food Chem. 77-81 (1975).

23. J. V. Pothuluri, J. P. Freeman, F. E. Evans, T. B. Moorman, C. E. Cerniglia, "Metabolism of Alachlor by the Fungus *Cunninghamella elegans*," J. Agric. Food Chem., **41**, 483-488 (1993).

24. R. K. Diefenbach, H. Keweloh, H.-J. Rehm, "Fatty Acids Impurities in Alginate Influence the Phenol Tolerance of Immobilized *Escherichia coli*," Appl. Microbiol. Biotechnol., **36**, 530-534 (1992).

25. A. B. Pepperman, J. W. Kuan, "Controlled Release Formulations of Alachlor Based on Calcium Alginate," J. Controlled Release, **34**, 17-23 (1995).

Received August 24, 1995
Accepted September 10, 1995
Manuscript 3952

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 661–677 (1996)

DETERMINATION OF EXPLOSIVES AND SOME METABOLITES OF TNT IN BIOLOGICAL AND ENVIRONMENTAL SAMPLES BY LIQUID CHROMATOGRAPHY ON A MIXED-MODE C_{18}-ANION COLUMN

John E. Caton, Wayne H. Griest

Chemical and Analytical Sciences Division
Oak Ridge National Laboratory
P.O. Box 2008; Building 4500-S
Oak Ridge, Tennessee 37830-6120

ABSTRACT

This method employs a ternary gradient generated from (1) a 90:10 water:methanol solution that is 0.015 M in potassium phosphate at pH 5.1; (2) methanol; and (3) acetonitrile to separate fifteen explosives, byproducts, and metabolites of 2,4,6-trinitrotoluene (TNT) on a C_{18}/anion exchange stationary phase. TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), six reduction metabolites and three oxidation metabolites of TNT have been separated and quantitated in a single chromatographic run. Because of the anion exchange character of the stationary phase, the anionic TNT metabolite 2,4,6-trinitrobenzoic acid also may be separated and quantitated using this method. Quantitation limits generally lower than 1 ppm have been established by a systematic certification procedure. The performance of the mixed mode separation method combined with diode array ultraviolet absorbance detection has been demonstrated with

samples derived from both biological (tissues and physiological fluids) and environmental (soil, composts, and leachates) sources.

INTRODUCTION

Remedial action and environmental restoration require fast and efficient methods for determining both explosives and their transformation products or metabolites. Methods that have been utilized for the determination of explosives are quite diverse including gas chromatography,[1] liquid chromatography.[2-6] liquid chromatography-mass spectrometry,[7-9] thin layer chromatography,[10] micellar electrokinetic capillary chromatography,[11] and supercritical fluid chromatography.[12-14] Reverse phase high performance liquid chromatography has emerged as the method most often used. However, the separation and determination problem becomes more complicated when an explosive compound such as 2,4,6-trinitrotoluene (TNT) is exposed to environmental or biological processes which lead to the formation of transformation products or metabolites.[15-17] Characterization of the metabolites resulting from environmental and/or biological action on both TNT[15-23] and dinitrotoluenes[24-30] have shown that nitrotoluenes are susceptible to both oxidation and reduction processes. Some of the known transformation products generated from TNT are listed in Table 1. The reduction process[17] involves attack on the nitro- function with the formation of a hydroxylamino-compound such as 4-hydroxylamino-2,6-dinitrotoluene (4OHA). This hydroxylamino compound, (4OHA), may dimerize to form 2,6,2',6'-tetranitro-4,4'-azoxytoluene (44'AT) or it may be further reduced to 4-amino-2,6-dinitrotoluene (4ADNT). The monoaminodinitro-compound may be further reduced to the corresponding diaminonitro-compound, 2,4-diamino-6-nitrotoluene (24DANT).

TNT is also susceptible to oxidation whereby the methyl group may be attacked to first form trinitrobenzyl alcohol (TNBOH), which can be further oxidized to trinitrobenzoic acid (TNBCOOH). Subsequently, the acid may be decarboxylated to form 1,3,5-trinitrobenzene (TNB).[31] Because the possible transformation products are both acidic and basic with a wide range of polarities it is difficult to develop a single procedure for the separation of all metabolites.[2,22] Compounds representing the range of TNT metabolites have been separated on a C_8 reverse phase column using a series of isocratic elutions with different acetonitrile:water mixtures.[22] In this present work a wide range of explosives and of TNT metabolites, (including the essentially anionic

Table 1

Listing of Abbreviations and Some Chromatographic Characteristics for Explosives and Some Metabolites of 2,4,6-Trinitrotoluene

COMPOUND (Abbreviation)	QL[1]	K[2]	A[3]
Explosives or ByProducts			
2,4,6-Trinitrotoluene (TNT)	0.2	5.36	1.22
Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)	0.4	4.47	1.30
Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)	0.4	6.51	1.25
2,4-Dinitrotoluene (24DNT)	0.4	6.11	1.21
2,6-Dinitrotoluene (26DNT)	0.5	6.08	1.24
1,3-Dinitrobenzene (13DNB)	0.2	4.12	1.23
Reduction Metabolites of TNT			
2-Amino-4,6-dinitrotoluene (2ADNT)	0.2	8.40	1.19
4-Amino-2,6-dinitrotoluene (4ADNT)	0.6	7.80	1.20
2,4-Diamino-6-nitrotoluene (24DANT)	0.7	1.84	1.37
2,6-Diamino-4-nitrotoluene (26DANT)	0.2	1.41	1.40
4-Hydroxyamino-2,6-dinitrotoluene (4OHA)	0.8	12.2	1.39
2,2'6,6'-Tetranitro-4,4'-azoxytoluene (44AT)	2.7	13.3	1.35
Oxidation Metabolites of TNT			
2,4,6-Trinitrobenzyl alcohol (TNBOH)	0.5	2.58	1.23
2,4,6-Trinitrobenzoic acid (TNBCOOH)	0.4	16.9	1.23
1,3,5-Trinitrobenzene (TNB)	0.2	3.18	1.32

[1] Quantitation Limit expressed as ppm concentration of injected sample when a 50 μL aliquot is injected, calculated per Hubaux and Vos (33).

[2]Capacity factor: ratio of retention time for given component to time to elute void volume from the system.

[3]Peak Asymmetry expressed as a ratio of the peak area after the peak maximum to the peak area before the maximum peak height.

metabolite, TNBCOOH) have been separated by a single gradient elution on a C_{18}/anion mixed mode column and determined using diode array UV absorbance detection.

MATERIALS

Analyses were carried out on a Hewlett-Packard Model 1090M Liquid Chromatograph equipped with a DR5 ternary solvent delivery system, a diode array ultraviolet (UV)-visible wavelength detector and the Hewlett-Packard 79994A ChemStation data system. This liquid chromatography system was fitted with an Alltech (Deerfield, IL) RP-C_{18}/Anion Mixed-Mode reverse phase/anion-exchange column (Product No. 72628) that was 150 mm X 4.6 mm. The mixed-mode support was bonded to spherical silica with a pore size of 100 Å and a particle size of 5 μM. The column cartridge was fitted with a matching guard cartridge (10 mm X 4.6 mm, Alltech No. 28013) containing the same mixed-mode packing.

Water and all organic solvents were "Baker Analyzed" HPLC reagent grade obtained from J. T. Baker, Inc. (Phillipsburg, NJ). 2ADNT, 4ADNT, 24DANT, 26DANT, and 13DNB were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). TNT, TNBCOOH, and TNB were purchased from ChemService (West Chester, PA). RDX (Lot No. 1130), HMX (Lot No. 1217), 24DNT (Lot No. 1147), and 26DNT (Lot No. 1148) were Standard Analytical Reference Materials (SARMS) obtained from the U. S. Army Environmental Center SARMS Repository (Aberdeen Proving Ground, MD). TNBOH, 44'AT, and 4OHA were synthesized in this laboratory by established procedures which are described elsewhere.[16] Monobasic and dibasic potassium phosphate were "Baker Analyzed" reagent obtained from J. T. Baker, Inc.

Except for standards prepared in HPLC-grade solvents, all samples were filtered through 0.μm nylon syringe filters that were 13 mm in diameter (Gelman No. 4427, Gelman Sciences, Ann Arbor, MI).

METHODS

The three eluting solutions for the ternary gradient were (A) 90:10 water:methanol (v/v) solution that was 0.015 M in potassium phosphate, (B)

methanol, and (C) acetonitrile. Eluting solution A was prepared by dissolving 4 grams (294 mmoles) of potassium dihydrogen phosphate in 1800 mL water and then adjusting the pH to 5.1 with approximately 0.8 mL of a 0.5 M stock solution of dipotassium hydrogen phosphate. Subsequently 200 mL of methanol were added causing the apparent pH to rise to 5.4. Other pH and phosphate concentrations in the 90:10 water:methanol eluting solution were prepared by adjusting the molar proportions and amounts of potassium dihydrogen phosphate and dipotassium hydrogen phosphate added to the water before adding the methanol.

Two gradient elution programs, which are summarized in Table 2, were developed for the C_{18}/Anion column. The longer gradient program (method 1, 45 minutes) starts by equilibrating the system with a mixture that contains 72% of the buffered water:methanol solution and 28 % acetonitrile. This equilibration eluent is continued for 1 min after the injection of a 50-µl sample aliquot. From 1 to 5 min a linear gradient reduces the proportion of the buffered water:methanol solution to 68% and increases the acetonitrile to 32%. Isocratic elution conditions are then maintained for the time interval from 5 to 14 min. Between 14 and 20 min a linear gradient reduces the buffered water:methanol solution by 4% per min and increases both methanol and acetonitrile by 2% per min so that at 20 min the eluting solution composition is 44% buffered water:methanol, 12 % methanol and 44% acetonitrile. From 20 to 26 min a linear gradient reduces the buffered water:methanol portion to 2% and increases methanol from 12% to 54% while maintaining the acetonitrile at 44%. Isocratic conditions are maintained from 26 to 33 min. From 33 to 38 min after injection a very steep gradient restores the eluting solution mixture to its initial conditions after which the column is re-equilibrated for 7 min.

The shorter ternary gradient program summarized in Table 2 (method 2) is 25 min in duration. This shorter program will work for sample mixtures that do not contain either TNBCOOH or interfering constituents that are strongly retained by the column.

Environmental solid samples such as soil were prepared by mixing one-gram portions with 4 mL of acetonitrile in a tightly capped vial and extracting for 18 hours in a cooled ultrasonic bath.[31] These extracts were then filtered through a 0.2 µm nylon membrane syringe filter before HPLC analysis. Aqueous leachates of composts were prepared according to the EPA SW-846 method 1312.[32] Organic extracts of tissue were also filtered in the same

Table 2

Ternary Gradient Programs for Elution of Explosives and TNT Metabolites on C_{18}/Anion Column with Flow Rate of 1mL/min

Method 1	% Buffer	% Methanol	%Acetonitrile	Method 2
0 min	72	0	28	0 min
1 min	72	0	28	0 min
5 min	68	0	32	5 min
14 min	68	0	32	11 min
20 min	44	12	44	15 min
26 min	2	54	44	18 min
33 min	2	54	44	21 min
38 min	72	0	28	22 min
45 min	72	0	28	25 min

manner following a preparation procedure described elsewhere[16] that involved tissue homogenization and ultrasonic cell disruption before the solvent extraction.

RESULTS AND DISCUSSION

The separation of a 15-component mixture of explosives and metabolites of TNT following the 45-minute gradient program is shown in Figure 1. To test the influence of the total concentration of phosphate in the 90:10 water:methanol component of this gradient the separation was carried out at three phosphate concentrations, (0.0075 M, 0.015 M and 0.03 M) during which the pH of the aqueous component was held constant at 5.1. The influence of pH was observed by holding the total phosphate concentration at 0.015 M while adjusting the pH of the aqueous component to 4.5, 5.1, 5.3, 5.7, and 6.4. Although the capacity factors for components other than TNBCOOH are not significantly influenced by pH and ionic strength, as illustrated in Figures 2 and 3, there is a major effect on peak asymmetry for some other components. In Figures 4 and 5 the influence of pH and phosphate concentration is shown for all components found to have a peak shape falling outside an asymmetry range between 0.9 and 1.4. The peak asymmetry for all 15 components is listed in Table 1 for pH 5.1 and a total phosphate concentration of 0.015 M. This was

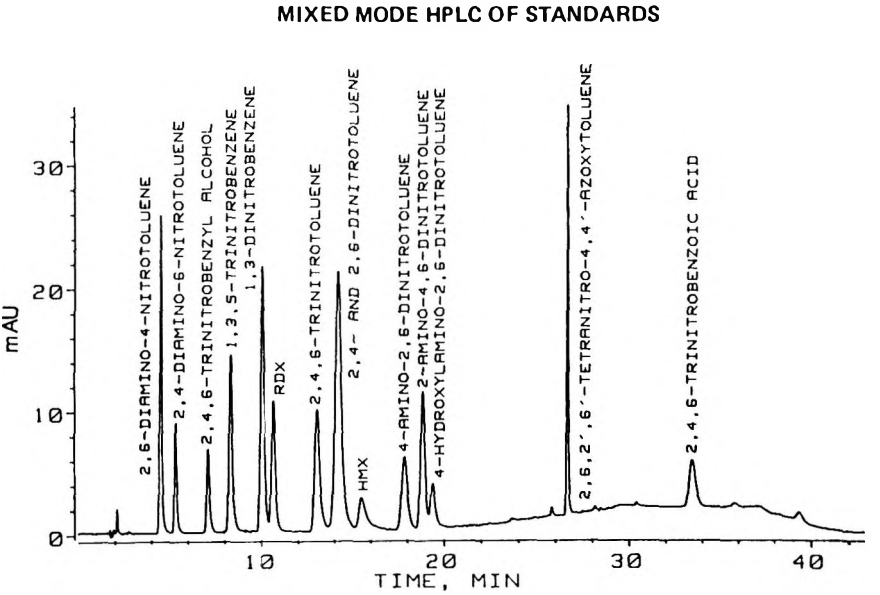


Figure 1. Chromatogram of explosives and TNT metabolites on C18/Anion column using the longer ternary gradient program summarized in Table 1. Chromatogram was monitored at 254 nm. Injection volume was 50 µL and the concentration of most components was 5 ppm.

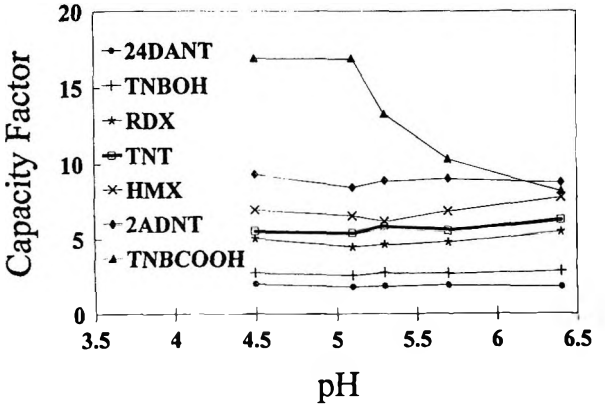


Figure 2. Plots showing the variation in retention with pH at a constant phosphate concentration of 0.015 M. Of all the compounds listed in Table 2 only TNBCOOH showed a significant trend between pH 4.5 and pH 6.4.

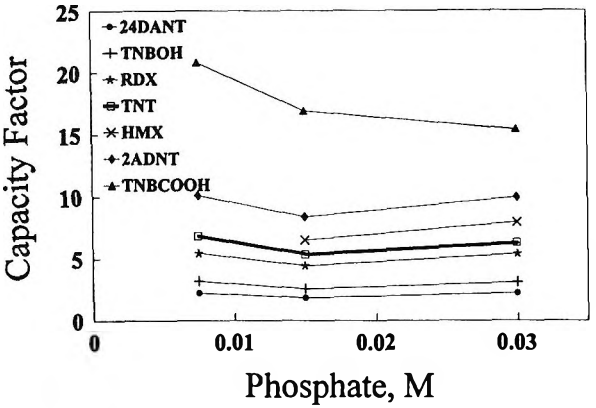


Figure 3. Plots showing the variation in capacity factor with changing total phosphate concentration at pH 5.1. Of all the compounds listed in Table 1 only TNBCOOH showed a significant trend toward lower retention as total phosphate concentration increased from 0.0075 M to 0.03 M.

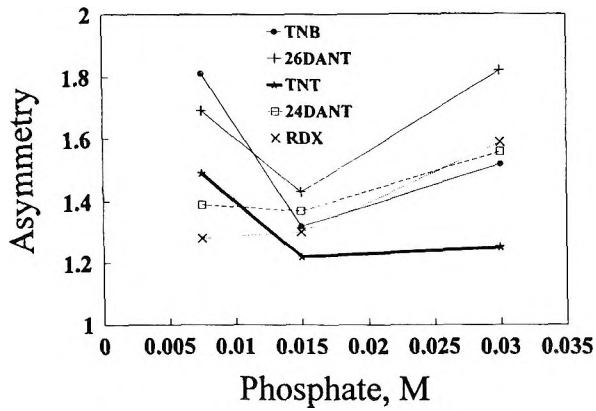


Figure 4. Plots showing the effect of total phosphate concentration on peak asymmetry. Only those compounds with peak asymmetry exceeding 1.4 at one or more of the phosphate concentrations tested are shown and compared to TNT.

the only set of conditions tested in which all components showed a peak asymmetry within the range of 1 to 1.4.

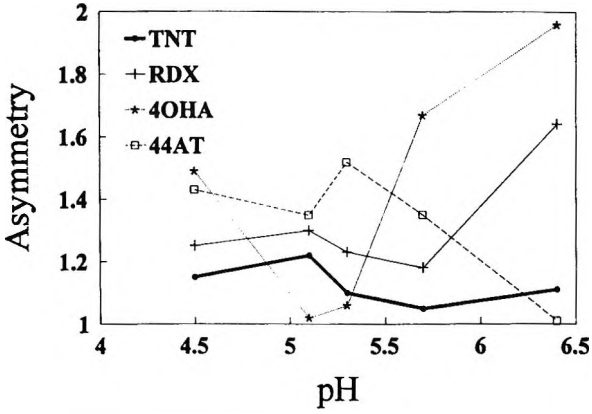


Figure 5. Plots showing the effect of pH on peak asymmetry. Only those compounds with peak asymmetry exceeding 1.4 at one or more of the pH levels tested are shown and compared to TNT.

The anion exchange character of the mixed-mode column would not appear to be a major factor in the retention of any of the tested components except TNBCOOH, for which significant changes in retention were observed for variations in both pH and total phosphate concentration . The mixed-mode column capacity for TNBCOOH was observed to be maximal at lower values of pH and lower total phosphate concentrations. Increasing phosphate concentrations at a given pH should decrease retention by the anion exchange sites on the column because of the increasing amount of anions (phosphate) available in the eluent to displace the anionic TNBCOOH. This same mass action effect of anions in the eluent also explains the decrease in retention with increasing pH at a constant total phosphate concentration. TNBCOOH is a relatively strong acid that should be almost completely ionized in very dilute solutions above pH 4.5. Therefore the fraction of TNBCOOH in the anionic form does not change significantly as pH increases above 4.5. On the other hand, at pH 4.5 the phosphate should be present almost exclusively in the singly ionized dihydrogen phosphate form. Between pH 4.5 and 6.5 the fraction of phosphate present in the doubly ionized hydrogen phosphate form increases by nearly two orders of magnitude.

In short, the total anionic eluting strength of a given total phosphate concentration increases substantially over the pH range tested. This assertion

that the anion exchange characteristics of the mixed mode column are principally responsible for the retention of the TNBCOOH is also supported by observations in other work. Yinon and Hwang[22] separated various groups of TNT metabolites including TNBCOOH using a series of isocratic elutions (acetonitrile:water eluents) on a C_8 column. In these reverse phase separations the TNBCOOH eluted very near the solvent front and always ahead of TNT, TNT metabolites (including 24DANT, 26DANT, 2ADNT, 4ADNT, 4OHA, and TNBOH), and even solvents such as acetone and methylene chloride.

Quantitation limits have been determined by the approach described by Hubaux and Vos[33] and are listed in Table 1 as sample concentrations when a 50 μL sample aliquot is injected. Quantitation was performed at 254 nm. The quantitation limits are generally 0.2 - 0.8 ppm. This 45-minute gradient method has also been certified for extracts and leachates of soils and composts using the U. S. Army Environmental Center's Quality Assurance Program Precertification and Certification Procedure.[34]

The performance of the mixed mode separation coupled with diode array UV absorbance detection is illustrated by its extensive application to the determination of explosives and TNT metabolites in soils contaminated with explosives, composts of those soils, aqueous leachates of the soils and composts,[35-36] and in tissues from animals living in highly contaminated sites.[16] In the soils, TNT, HMX and RDX were the main contaminants detected. During composting, the rapid biotransformation of TNT led to the early appearance of its reduction metabolites. The 2A46DNT and 4A26DNT were observed in both the fresh composts and their leachates. The 24DA6NT and 26DA4NT grew in as the monoaminonitrotoluenes decreased in abundance during the course of composting, and then decreased as composting continued. Except for an azoxy dimer (44'AT) in leachates prepared from soils in the early stages of composting, no other transformation products or reduction/oxidation metabolites of TNT (or HMX and RDX) were observed.

Figure 6 shows the chromatograms (using the shorter gradient program) for the leachates and acetonitrile extract of a soil after composting for 5 days. HMX, RDX, TNT, and its metabolites are clearly separated and free of detectable interferences. The azoxy dimer, 44'AT, was of particular interest because it has only very rarely been observed. It was observed only in the leachate, suggesting that the 18-hr tumbling in water at room temperature promoted further reactions of TNT metabolites present in the soil compost. The dimer was identified by its retention time and UV spectrum (Figure 7A) in

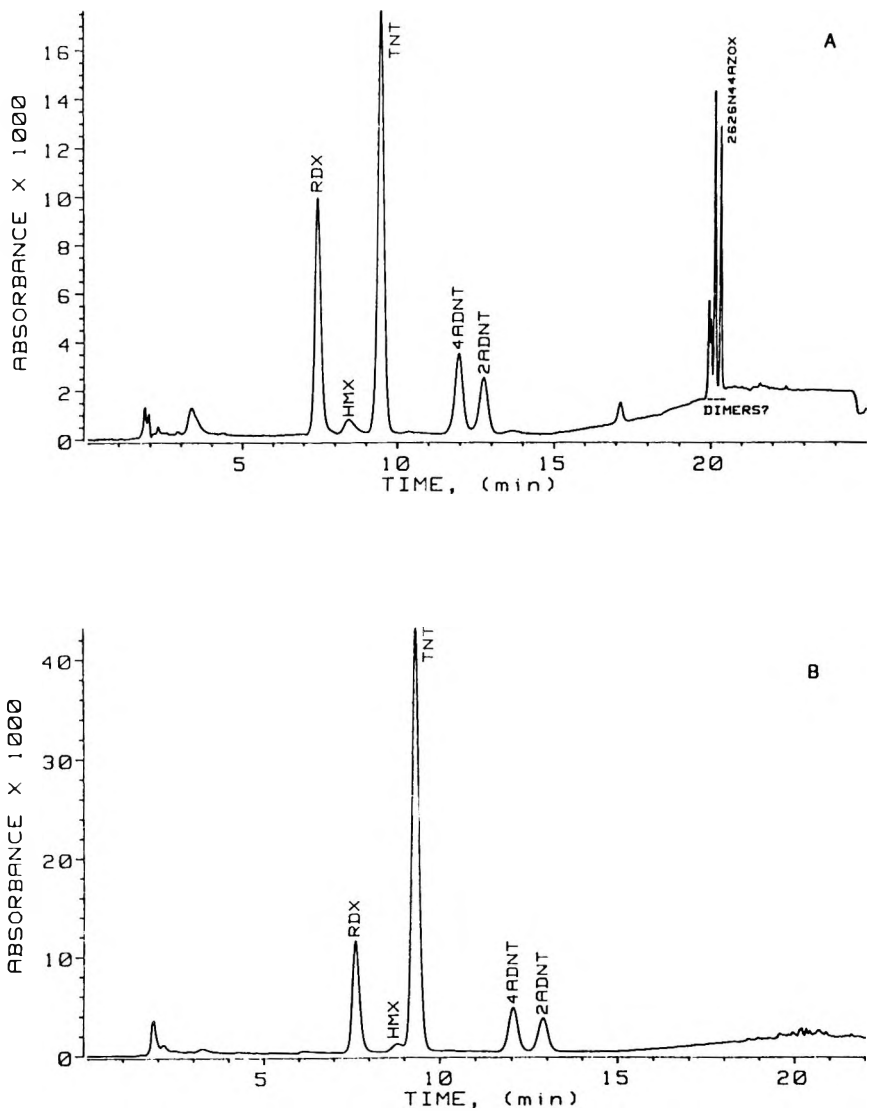


Figure 6. Chromatograms of explosives and TNT metabolites in soil compost leachate (A) and acetonitrile extract (B) on C_{18}/Anion column using the shorter ternary gradient program (method 2 in Table 2).

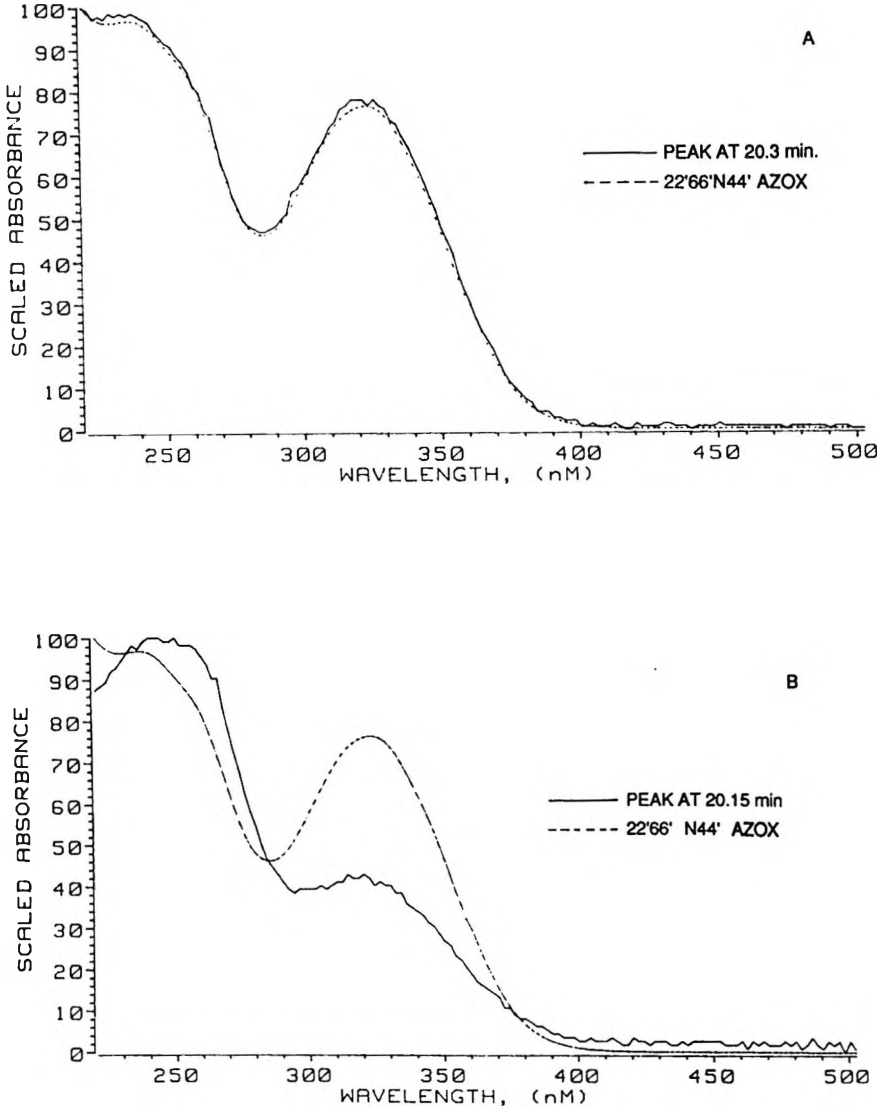


Figure 7. Comparison of the spectrum of authentic 44'AT with HPLC peaks eluting at (A) 20.30 min and (B) 20.15 min. in the chromatogram of the leachate from the soil compost (Figure 6).

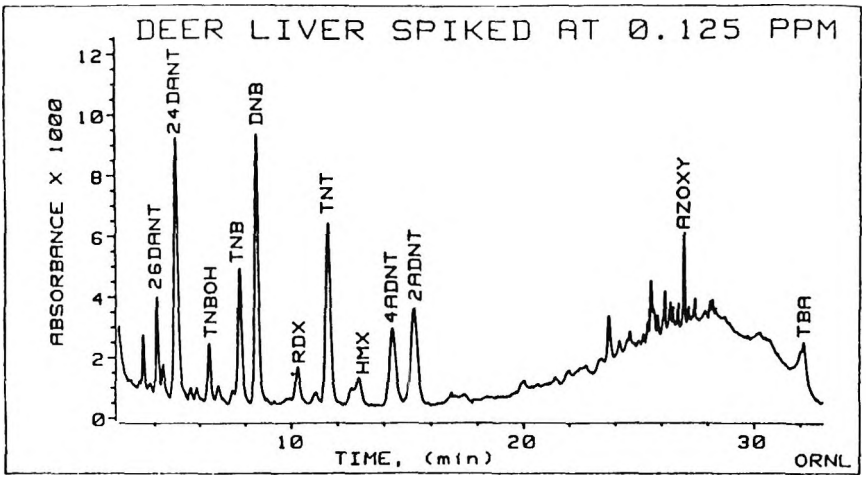


Figure 8. Chromatogram of explosives and metabolites extracted from a deer liver sample which had been spiked with authentic standards at a concentration of 0.125 ppm each. The longer ternary gradient program (method 1 in Table 2) was used.

the leachate. The peak eluting immediately before 44'AT at ca. 21.7 min is suspected to also be an azoxydimer because its spectrum was almost identical to that of 44'AT but shifted a few nm (Figure 7B). The other two peaks in the quartet could be azoxydimers; a total of four isomers (two symmetrical and two unsymmetrical isomers) appears possible in the microbial metabolism of TNT.

TNBCOOH has not yet been observed in any sample extract, most likely because this compound is readily decarboxylated in solvents such as ethanol and dioxane where the carboxylate ion is not as strongly solvated as it is in aqueous solution.[37] In this study TNBCOOH standards in water solution stored at 4°C were observed to be stable for many months, whereas TNBCOOH began to disappear from a methanol solution within a few hours and from an acetonitrile solution within 30 min. It appears likely that common sample preparation methods in which explosives and metabolites are extracted from aqueous-based biological and environmental samples into organic solvents would show increasingly diminished amounts of TNBCOOH as the preparation and storage time before analysis increased.

The utility of the method for analysis of animal tissue extracts is

illustrated by the chromatogram in Figure 8. The chromatogram shows the separation of an extract from a deer liver tissue sample spiked with TNT and its metabolites at 0.125 ppm. Although the levels are near the limit of detection, the peaks are clearly visible and identifiable. In this study, neither TNT nor its soluble free metabolites were detected in any sample at a limit of 0.2 ppm.[16] It was suspected that the metabolites were mainly highly polar, water-soluble forms which eluted in the solvent peak, and also conjugates which would not be detected without performing hydrolysis before the extraction.

CONCLUSIONS

The mixed mode combination of anion exchange and reverse phase mechanisms achieves a highly efficient separation of explosives and also both reduction and oxidation metabolites of TNT in a single chromatographic run. Coupling this separation mechanism with diode array UV absorbance detection allows the confident analysis of extracts and leachates of soils and extracts of animal tissues.

ACKNOWLEDGEMENTS

This research was sponsored by the U.S. Army Biomedical Research and Development Laboratory and the U.S. Army Environmental Center, under U.S. Department of Energy Contract DE-AC05-84-OR-21400 with Martin Marietta Energy Systems, Inc.

REFERENCES

1. R. J. Spanggord, B. W. Gibson, R. G. Keck, D. W. Thomas, Environ. Sci. Technol., **16**, 229-232 (1982).

2. D. L. Kaplan, A. M. Kaplan, Anal. Chim. Acta, **136**, 425-428 (1982).

3. M. Emmrich, M. Kaiser, H. Ruden, S. Sollinger, J. Chromatogr., **645**, 89-94 (1993).

4. J. B. F. Lloyd, Adv. Chromatogr., **32**, 173-261 (1992).

5. M. Walsh, T. F. Jenkins, Anal. Chim. Acta, **231**, 313-315 (1990).

6. F. Ahmad, D. J. Roberts, J. Chromatogr., **693**, 167-175 (1995).

7. J. Yinon, D.-G. Hwang, J. Chromatogr., **268**, 45-53 (1983).

8. J. Yinon, D.-G. Hwang, J. Chromatogr., **394**, 253-257 (1987).

9. R. D. Voyksner, J. Yinon, J. Chromatogr., **354**, 393-405 (1986).

10. C. Steuckart, E. Berger-Preiss, K. Levsen, Anal. Chem., **66**, 2570-2577 (1994).

11. W. Kleibohmer, K. Cammann, J. Robert, E. Mussenbrock, J. Chromatogr. **638**, 349-356 (1993).

12. J. Via, L. T. Taylor, Anal. Chem., **66**, 1385-1395 (1994).

13. A. Munder, S. N. Chesler, S. A. Wise, J. Chromatogr., **521**, 63-70 (1990).

14. W. H. Griest, C. Guzman, M. Dekker, J. Chromatogr., **467**, 423-429 (1989).

15. B. H. Woollen, M. G. Hall, R. Craig, G. T. Steel, British J. Ind. Medicine, **43**, 465-473 (1986).

16. L. R. Shugart, W. H. Griest, E. Tan, C. Guzman, J. E. Caton, C.-H. Ho, B. A. Tomkins, **TNT Metabolites in Animal Tissues**, ORNL/Tm 1336, Oak Ridge National Laboratory, Oak Ridge, TN (Dec., 1990, published June, 1990).

17. D. L. Kaplan, A. M. Kaplan, Appl. Environ. Microbiol., **44**, 757-760 (1982).

18. W. E. Pereira, D. L. Short, D. B. Manigold, P. K. Roscio, Bull. Environm. Contam. Toxicol., **21**, 554-562 (1979).

19. J. Yinon, D.-G. Hwang, Biomed. Chrom., **1**, 123-125 (1986).

20. J. Almog, S. Kraus, A. Basch, Arch. Toxicol., **Suppl. 6**, 351-353 (1983).

21. J. Yinon, D.-G. Hang, J. Chromatogr., **375**, 154-158 (1986).

22. J. Yinon, D.-G. Hwang, J. Chromatogr., **339**, 127-137 (1985).

23. J. Yinon, D.-G. Hwang, Toxicology Letters, **26**, 205-209 (1985).

24. J. A. Bond, D. E. Rickert, Drug Metab. and Dispos., **9**, 10-15 (1981).

25. D. Guest, S. R. Schnell, D. E. Rickert, J. G. Dent, Toxicol. Appl. Pharmacol., **64**, 160-168 (1982).

26. M. Mori, T. Matsuhashi, T. Miyahara, S. Shibata, C. Izima, H. Kozuka, Toxicol. Appl. Pharmacol., **76**, 105-112 (1984).

27. D. E. Rickert, R. M. Long, Drug Metab. and Dispos., **9**, 226-232 (1981).

28. D. E. Rickert, R. M. Long, Toxicol. Appl. Pharmacol., **56**, 286-293 (1980).

29. H. A. J. Schut, R. Dixit, T. R. Loeb, G. D. Stoner, Biochem. Pharmac., **34**, 969-976 (1985).

30. M. J. Turmner, Jr., R. J. Levine, D. D. Nystrom, Y. S. Crume, D. E. Rickert, Toxicol. Appl. Pharmacol., **80**, 166-174 (1985).

31.T. F. Jenkins, C. E. Grant, Anal. Chem., **59**, 1326-1331 (1987).

32. U. S. Environmental Protection Agency, Office of Solid Waste and Emergency Response, **Test Methods for Evaluating Solid Waste, SW-846**, first Update, U. S. Environmental Protection Agency, Washington, D.C. (November, 1990).

33. A. Hubaux and G. Vos, Anal. Chem., **42**, 849-855 (1980).

34. USATHAMA, USER'S MANUAL, (Prepared by Potomac Research, Inc., Aberdeen Proving Ground, MD) The Installation Restoration Project Quality Assurance Programs, U.S. Army Toxic and Hazardous Materials Agency, Aberdeen Proving Ground, MD, June, 1988.

35. W. H. Griest, A. J. Stewart, R. L. Tyndall, J. E. Caton, C.-H. Ho, K. S. Ironside, W. M. Caldwell, E. Tan, Environ. Tox. Chem., **12**, 1105-1116 (1993).

36. W. H. Griest, R. L. Tyndall, A. J. Stewart, J. E. Caton, A. A. Vass, C.-H. Ho, W. M. Caldwell, Environ. Tox. Chem., **14**, 51-59 (1995).

37. P. Segura, J. F. Bunnett, L. Villanova, J. Org. Chem., **50**, 1041-1045 (1985).

Received August 30, 1995
Accepted September 18, 1995
Manuscript 3955

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 679 (1996)

ANNOUNCEMENT

CONGRATULATIONS

It is with great pleasure that we congratulate Dr. Ram Gupta, world renowned scientist, upon his retirement from a most distinguished, productive and focused career as Head of Toxicology at the St. Joseph's Hospital in Hamilton, Ontario, Canada. Dr. Gupta is also a highly regarded scholar in the field of chromatography, specializing in biomedical applications of LC science and technology.

After receiving his Ph.D. in Chemistry at McMaster University, he served as a post doctoral research fellow. In 1971, he joined the St. Joseph's Hospital as assistant chemist. Later, he assumed the position of Head, Toxicology.

Dr. Gupta has served on the Editorial Board of the Journal of Chromatography (Biomedical Applications), the Journal of Liquid Chromatography, and as *ad hoc* reviewer for grant applications for US and Canadian agencies, and as invited speaker in many regional and national meetings.

In sharing his research findings, Dr. Gupta has continuously contributed to the scientific literature, with three additional articles coming after his retirement. From the many meetings I had with Ram, I have always been impressed by his sincerity and pleasant demeanor.

Dr. Gupta's career will undoubtedly inspire younger scientists in the future. It is, thus, fitting to pay tribute to Dr. Ram Nath Gupta, as a friend, and colleague and, on behalf of the scientific community, to wish him all the best upon his retirement.

Dr. Steven H. Wong
Medical College of Wisconsin

J. LIQ. CHROM. & REL. TECHNOL., 19(4), 681–685 (1996)

LIQUID CHROMATOGRAPHY CALENDAR

1996

FEBRUARY 5 - 7: PrepTech'96, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. R. Stevenson, 3338 Carlyle Terrace, Lafayette, CA 94549, USA, or Ms. Joan Lantowski, ISC Technical Conferences, Inc., 30 Controls Drive, P. O. Box 559, Shelton, CT 06484, USA.

FEBRUARY 25 - 29: AIChE Spring National Meeting, Sheraton Hotel, New Orleans, Louisiana. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

MARCH 3 - 8: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

MARCH 24 - 29: 211th ACS National Meeting, New Orleans, LA. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography and Extraction, Indianapolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MARCH 31 - APRIL 4: 7th International Symposium on Supercritical Fluid Chromatography & Extraction, Indianpolis, Indiana. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

APRIL 17 - 19: VIIth International Symposium on Luminescence

Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Université de Nice, Fracnce. Contact: Prof. W. R. G. Baeyens, University of Ghent Pharmaceutical Institute, Lab or Drug Quality Control, Harelbekestraat 72, Ghent, Belgium. Email: willy.baeyens@rug.ac.be.

MAY 7 - 9: VIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis - Detection Techniques and Applications in Chromatography and Capillary Electrophoresis, Monte Carlo, Monaco. Contact: Prof. Willy R. G. Baeyens, University of Ghent, Pharmaceutical Institute, Harelbekestraat 72, B-9000 Ghent, Belgium.

JUNE 16 - 21: "HPLC '96: Twentieth International Symposium on High Performance Liquid Chromatography," San Francisco Marriott Hotel, San Francisco, California. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JULY 1 - 3: International Symposium on Polymer Analysis and Characterization, Keble College, Oxford University, U.K. Contact: Prof. J. V. Dawkins, Dept. of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.

JULY 14 - 18: 5th World Congress of Chemical Engineering, Marriott Hotel, San Diego, California. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 9 - 14: 31st Intersociety Energy Conversion Engineering Conference (co-sponsored with IEEE), Omni Shoreham Hotel, Washington, DC. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 17 - 20: 31st National Heat Transfer Conference, Westin Galleria, Houston, Texas. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

AUGUST 18 - 23: 212th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 1 - 6: 11th Symposium on Quantitative Structure-Activity Relationships: Computer-Assisted Lead Finding and Optimization,"

Lausanne, Switzerland. Contact: Dr. Han van de Waterbeemd, F. Hoffmann-La Roche Ltd., Dept PRPC 65/314, CH-4002 Basle, Switzerland.

SPETEMBER 7: Field-Flow Fractionation Workshop VIII, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 11: Sixth International Symposium on Field-Flow Fractionation, Ferrara, Italy. Contact: F. Dondi, Dept of Chem, University of Ferrara, via L. Borsari 46, I-44100 Ferrara, Italy.

SEPTEMBER 9 - 12: Saftey in Ammonia Plants & Related Facilities, Westin at Copley Place, Boston, Massachusetts. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

OCTOBER 24 - 26: 52nd Southwestern Regional Meeting, ACS, Houston, Texas. Contact: J. W. Hightower, Chem Eng Dept, Rice Univ, Houston, TX 77251, USA.

NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

1997

APRIL 6 - 11: 213th ACS National Meeting, San Antonio, Texas. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

APRIL 14 - 19: Genes and Gen Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

SEPTEMBER 7 - 12: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1998

MARCH 29 - APRIL 3: 215th ACS National Meeting, St. Louis, Missouri. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 23 - 28: 216th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

1999

MARCH 21 - 26: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 22 - 27: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2000

MARCH 26 - 31: 219th ACS National Meeting, Las Vegas, Nevada.

Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 20 - 25: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2001

APRIL 1 - 6: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

AUGUST 19 - 24: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

2002

APRIL 7 - 12: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 13: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

The **Journal of Liquid Chromatography and Related Technologies** will publish, at no charge, announcements of interest to scientists in every issue of the journal. To be listed in the Liquid Chromatography Calendar, we will need to know:

a) Name of the meeting or symposium,
b) Sponsoring organization,
c) When and where it will be held, and
d) Whom to contact for additional details.

Incomplete information will not be published. You are invited to send announcements to **Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 2180, Cherry Hill, NJ 08034-0162, USA.**

INSTRUCTIONS TO AUTHORS

The *Journal of Liquid Chromatography & Related Technologies* is published in the English language for the rapid communication of research results in liquid chromatography and its related sciences and technologies.

Directions for Submission

One complete original manuscript and two (2) clear copies, with figures, must be submitted for peer review. After all required revisions have been completed, and the final manuscript has been accepted, the author will be asked to provide, if possible, a 3½" or 5¼" PC-Compatible computer diskette containing the complete manuscript. Microsoft Word, Word for Windows, WordPerfect, WordPerfect for Windows and ASCII are preferred formats. Text, including tables, and figures, if in electronic format, should be saved in separate files on the diskette. Label the diskette with the corresponding author's last name, the title of the manuscript and the file number assigned to the manuscript.

Submission of a manuscript on diskette, in a suitable format, will significantly expedite its publication.

Manuscripts and computer diskettes should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography & Related Technologies
P. O. Box 2180
Cherry Hill, NJ 08034-0162

Reprints

Due to the short production time for papers in this journal, it is essential to order reprints immediately upon receiving notification of acceptance of the manuscript. A reprint order form will be sent to the author with the letter of acceptance for the manuscript. Reprints are available in quantities of 100 and multiples thereof. Twenty (20) free reprints will be included with orders of 100 or more reprints.

Format of the Manuscript

NOTE: Failure to adhere to the following guidelines will delay publication of a manuscript.

1. The preferred dimensions of the printed area of a page are
   6" (15.2 cm) width by 8.5" (21.6 cm) height.
   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

Title
Author(s)' names and full addresses
Abstract
Text Discussion
References

2. **Title & Authors**: The entire title should be in bold-face capital letters and centered within the width of the printed area, located 2 inches (5.1 cm) from the top of the page. This should be followed by 2 lines of space, then by the names and addresses of the authors, also centered, in the following manner:

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN WATER BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce, H. R. Brown
Utah Biomedical Test Laboratory
520 Wakara Way
Salt Lake City, Utah 84108

3. **Abstract**: The heading **ABSTRACT** should be typed boldface, capitalized and centered, 2 lines below the addresses. This should be followed by a single-spaced, concise abstract. Allow 2 lines of space below the abstract before beginning the text of the manuscript.

4. **Text Discussion**: Whenever possible, the text discussion should be divided into major sections such as

INTRODUCTION
MATERIALS
METHODS
RESULTS
DISCUSSION
ACKNOWLEDGMENTS

These **major headings** should be separated from the text by two lines of space above and one line of space below. Each major heading should be typed boldface, in capital letters, centered.

Secondary headings, if any, should be placed flush with the left margin, and have the first letter of main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. The **first line of each paragraph** within the body of the text should be indented a half inch.

6. **Acknowledgments**, sources of research funds and address changes for authors should be listed in a separate section at the end of the manuscript, immediately preceding the references.

7. **References** should be numbered consecutively and placed in a separate section at the end of the manuscript. They should be typed single-spaced, with one line space between each reference. Each reference should contain names of all authors (with initials of their first and middle names); do not use *et al.* for a list of authors. Abbreviations of journal titles will follow the American Chemical Society's Chemical Abstracts List of Periodicals. The word **REFERENCES**, in boldface type, should be capitalized and centered above the reference list.

Following are acceptable reference formats:

Journal:

1. D. K. Morgan, N. D. Danielson, J. E. Katon, Anal. Lett., 18, 1979-1998 (1985).

Book:

1. L. R. Snyder, J. J. Kirkland, **Introduction to Modern Liquid Chromatography**, John Wiley & Sons, Inc., New York, 1979.

Chapter in a Book:

1. C. T. Mant, R. S. Hodges, "HPLC of Peptides," in **HPLC of Biological Macromolecules**, K. M. Gooding, F. E. Regnier, eds., Marcel Dekker, Inc., New York, 1990, pp. 301-332.

8. Each page of manuscript should be numbered lightly, with a light blue pencil, at the bottom of the page.

9. Only standard symbols and nomenclature, approved by the International Union of Pure and Applied Chemistry (IUPAC) should be used. **Hand-drawn characters are not acceptable.**

10. Material that cannot be typed, such as Greek symbols, script letters and structural formulae, should be drawn carefully with dark black India ink. Do not use any other color ink.

Additional Typing Instructions

1. The manuscript must be prepared on **good quality white bond paper**, measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). International paper, size A4 is also acceptable. The typing area of the first page, including the title and authors, should be 6" (15.2 cm) wide by 8.5" (21.6 cm) height.

2. All text should be **typed single-spaced**.

3. It is essential to use **dark black** typewriter or printer ribbon so **that clean, clear, solid characters** are produced. Characters produced with a dot/matrix printer are not acceptable, even if they are "near letter quality" or "letter quality." Erasure marks, smudges, hand-drawn corrections and creases are not acceptable.

4. **Tables** should be typed as part of the text, but in such a way as to separate them from the text by a 2-line space above and below the table. Tables should be inserted in the text as close to the point of reference as possible. **A table may not be longer than one page**. If a table is larger than one page, it should be divided into more than one table. The word **Table** (followed by an Arabic number) should precede the table and should be centered above the table. The title of the table should have the first letters of all main words in capitals. Table titles should be typed single line spaced, across the full width of the table.

5. **Figures** (drawings, graphs, etc.) should be professionally drawn in black India ink on separate sheets of white paper, and should be placed at the end of the text. They should not be inserted into the body of the text. They should not be reduced to a small size. Preferred size for figures is from 5 inches x 7 inches (12.7 cm x 17.8 cm) to 8½ inches by 11 inches (21.6 cm x 27.9 cm). Photographs should be professionally prepared, black and white, *glossy* prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn.

Captions for figures should be typed single-spaced on a separate sheet of white paper, along the full width of the type page, and should be preceded with the

word **Figure** and an Arabic numeral. All figures and lettering must be of a size that will remain legible after a 20% reduction from the original size. Figure numbers, name of senior author and an arrow indicating "top" should be written in light blue pencil on the back of the figure. Indicate the approximate placement for each figure in the text with a note written with a light blue pencil in the margin of the manuscript page.

6. The reference list should be typed single-spaced. A single line space should be inserted after each reference. The format for references should be as given above.

Manuscripts which require correction of English usage will be returned to the author for major revision.

Create polymer products that have an optimal molecular weight distribution with the...

Handbook of Size Exclusion Chromatography

(Chromatographic Science Series/69)

edited by **CHI-SAN WU**
International Specialty Products, Wayne, New Jersey

January, 1995 / 472 pages, illustrated / $175.00

This outstanding single-source reference details the **practical** use of size exclusion chromatography (SEC) in characterizing the molecular weight distribution of important polymeric materials—addressing problems encountered in the SEC of specific substances, including copolymers, polyamides, polyvinyl alcohol and acetate, lignin derivatives, proteins, and starch.

Written by more than **25** internationally renowned authorities in their respective fields, the ***Handbook of Size Exclusion Chromatography***

- delineates the employment of semirigid polymer gels and modified silica-based packing materials for SEC
- discusses recently developed molecular weight-sensitive detectors
- examines column technology in SEC
- covers materials seldom considered in SEC surveys such as rubber and asphalt
- describes how to determine the absolute molecular distribution of polymers by SEC without calibration
- and more!

Furnishing over **1000** citations to key sources in the literature as well as more than **275** useful tables, drawings, and equations, the ***Handbook of Size Exclusion Chromatography*** is an invaluable resource for chromatographers; analytical, polymer, organic, and physical chemists; biochemists; quality assurance managers; and upper-level undergraduate and graduate students in these disciplines.

Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
(212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
Tel 061-261-8482

Contents

ISBN: 0—8247—9288—2

This book is printed on acid-free paper.

Explore the latest advances in the field of...

Ion Exchange and Solvent Extraction

VOLUME 12

Offers **easy access** to Russian and Chinese source material previously unavailable to Western researchers!

edited by

JACOB A. MARINSKY
State University of New York at Buffalo

YIZHAK MARCUS
Hebrew University of Jerusalem, Israel

March, 1995
456 pages, illustrated
$195.00

Volume 12 of this unique series continues in the tradition of its widely received predecessors, presenting **current** developments in the theory and practice of ion exchange.

This authoritative reference provides interdisciplinary coverage of contemporary topics, including

- the nature of metal-ion interaction with oppositely charged sites of ion exchangers
- high-pressure ion-exchange separation of rare earth elements
- the commercial recovery of valuable minerals from seawater and brines by ion exchange and sorption
- the kinetics of ion exchange in heterogeneous systems
- the ion-exchange equilibria of amino acids
- and much more!

The ***Ion Exchange and Solvent Extraction Series*** treats ion exchange and solvent extraction both as discrete topics and as a unified, multidisciplinary study—presenting new insights for researchers in many chemical and related fields. The volumes in this now classic series are of major importance to analytical, coordination, process, separation, surface, organic, inorganic, physical, and environmental chemists; geochemists; electrochemists; radiochemists; biochemists; biophysicists; hydrometallurgists; membrane researchers; and chemical engineers.

Contents

ISBN: 0—8247—9382—X

This book is printed on acid-free paper.

Marcel Dekker, Inc.
270 Madison Avenue
New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812
CH-4001 Basel, Switzerland
Tel. 061-261-8482

LIQUID CHROMATOGRAPHY FOR THE ANALYST

RAYMOND P. W. SCOTT
Georgetown University, Washington, D.C., and Birbeck College, University of London, United Kingdom

January, 1994
344 pages, illustrated
$75.00

This practical guide provides a clear presentation of the chromatographic process—demonstrating the functions of all associated instrumentation *and* the procedures necessary to obtain accurate qualitative and quantitative results.

Supplies a* host of applications *from a variety of sources to help identify the best equipment, the most appropriate columns, and the most suitable phase systems for specific samples!

Written by an international expert with over 45 years of industrial and academic experience, ***Liquid Chromatography for the Analyst***

- covers essential fundamental theory
- explains the chromatographic process using established physical chemical terminology
- illustrates chromatographic behavior with current practices
- gives **useful examples** to aid in applying principles to actual problems
- furnishes supporting experimental evidence for theoretical explanations
- and much more!

With its direct, jargon-free style that permits easy access to information, ***Liquid Chromatography for the Analyst*** is an invaluable resource for analytical chemists, laboratory technicians, and upper-level undergraduate, graduate, and continuing-education courses in analytical chemistry or separation science.

CONTENTS

ISBN: 0-8247-9184-3
This book is printed on acid-free paper.

MARCEL DEKKER, INC.

270 Madison Avenue
New York, NY 10016
(212) 696-9000

Hutgasse 4, Postfach 812
CH-4001 Basel, Switzerland
Tel. 061-261-8482

Keep state-of-the-art theoretical and applied research developments at your fingertips with...

Advances in Chromatography

Volume 36

edited by

PHYLLIS R. BROWN
University of Rhode Island, Kingston

ELI GRUSHKA
Hebrew University of Jerusalem, Israel

October, 1995 / 464 pages, illustrated / $175.00

Reviewer praise for previous editions...

"...reflects the high standards expected from this respected series."
—Clinical Chemistry

"...the articles [are] of high scientific standard, up to date, very well written, and interesting to read...well presented and edited."
—Journal of Chromatography

"...a valuable contribution to the chromatography literature... belongs in every library used by chromatographers."
—Liquid Chromatography

"...maintains the high quality that chromatographers have come to expect from this invaluable series."
—Journal of Pharmaceutical Sciences

The rapidly expanding growth of the literature on chromatography, capillary electrophoresis, field flow fractionation, and other separation techniques makes it difficult for any individual to maintain a coherent view of progress in the field. Rather than attempt to read the avalanche of original research papers, investigators trying to preserve even a modest awareness of advances must rely upon **authoritative surveys.**

Featuring reliable, **up-to-the-minute reviews** of major developments in separations, this critically praised series separates the most important advances from an overabundance of supplementary materials.

Internationally acclaimed experts analyze the most current innovations in their areas of specialization!

Providing more than 850 bibliographic citations, allowing for further, in-depth study of recent trends in research, **Volume 36** examines timely subjects such as

- multilinear regression, canonical correlation, and factor and principal component methods of analysis in the evaluation of retention data matrices
- molecular recognition mechanisms in the liquid chromatographic separation of fullerenes
- the latest techniques in the use of capillary electrophoresis and mass spectrometry for sequencing antisense oligonucleotides
- analytical tools and separation methods for the analysis of drugs of abuse in biological fluids
- the applications of high-performance liquid chromatography coupled with nuclear magnetic resonance spectroscopy
- and much more!

Advances in Chromatography is an indispensable resource for all researchers who need to use separation methods effectively—especially analytical, organic, inorganic, clinical, and physical chemists; chromatographers; biochemists and biological chemists; agrochemists; chemical, materials, pollution, and quality control engineers; biotechnologists; toxicologists; pharmacologists; pharmacists; physiologists; zoologists; botanists; food, cosmetic, polymer, and environmental scientists; microbiologists; virologists; oceanographers; research and quality control scientists in academia, government, hospitals, and industry; and upper-level undergraduate and graduate students in these disciplines.

CONTENTS

ISBN: 0—8247—9551—2

This book is printed on acid-free paper.

MARCEL DEKKER, INC.

270 Madison Avenue, New York, NY 10016 • (212) 696-9000
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland • Tel. 061-261-8482

J. LIQ. CHROM. & REL. TECHNOL., 19(4), (1996)

Contents Continued

JOURNAL OF LIQUID CHROMATOGRAPHY
& RELATED TECHNOLOGIES

Volume 19, Number 4, 1996

CONTENTS

(continued on inside back cover)

MARCEL DEKKER, INC. New York, Basel, Hong Kong
Contributions to this journal are published free of charge