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SEPARATION AND DETERMINATION OF AUXINS BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

In this paper, the separation of fifteen plant auxins by capillary electrophoresis was investigated. A simple and efficient method was developed. The migration behaviour of the auxins under different separation conditions was studied. Optimum separation was obtained with cyclodextrin-modified borate-phosphate buffer. This method was applied to the determination of indole acetic acid (IAA), a well known auxin in plant samples, with cinnamic acid as the internal standard. The method is linear over the range of 0.4 - 8.0 mg/mL, with 82.4% recovery and a limit of quantitation of 0.4 mg/mL.

INTRODUCTION

Auxins are a group of plant hormones which play important roles in a variety of diverse plant growth and plant development responses, such as cell elongation, cell division and cell differentiation.[1] Among the auxins, indole-3-acetic acid (IAA) is the most ubiquitous compound in plants.[1]

Other natural auxins include indole acids, such as indole-3-butyric acid (IBA)[2] and indole-3-propionic acid (IPA),[3] various indole acid conjugates, and phenylacetic acid (PAA).[4] Synthetic auxins include 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorphenoxyaceticacid (2,4,5-T), 2,3,5-triiodobenzoic acid (TIBA), α-naphthalenic acid (NAA),[5] etc.

A commonly used method for the detection of novel plant hormones is bioassay, but the choice of suitable bioassays can be problematical, and it is now generally accepted to be unsuitable for quantitative work. In addition, several other methods have been reported for the determination of auxins, including high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS),[6] and electroanalytical techniques.[7] As for the determination of auxins in plant samples, HPLC with fraction collection is generally used to purify the extracted samples before measurements. GC-MS is then used to identify the compounds, and the best method for the quantitive analysis is to use heavy isotope-labelled internal standards.[9-11] Although very precise and accurate results have been obtained by such methods, many special or costly items and chemicals such as HPLC preparative columns, a mass spectrometric detector and radioactive compounds are needed. Furthermore, the column is easily contaminated, and it takes a long time to perform the analysis. To date, there have been few papers concerning the separation of plant hormones by capillary electrophoresis, although this technique is rapidly gaining popularity due to its potential to achieve very high separation efficiencies.[8]

In this paper, the effects of different separation conditions on migration time, separation efficiency and resolution in the analysis of auxins were examined. An efficient and simple method for the separation of fifteen auxins by capillary electrophoresis (CE) was developed. The method was applied in the separation and quantitative analysis of IAA in shoot apices of Vanda Miss Joaquim, a species of orchid.

MATERIALS AND METHODS

Reagents and Materials

The fifteen auxins (See Fig. 1) used were purchased from Sigma Chemical Co. (St. Louis, USA). Shoot apices of Vanda Miss Joaquim were provided by Professor Chong-Jin Goh, Botany Department of National University of Singapore (Singapore). Methanol was purchased from Fisher Scientific (Pittsburgh, USA). Ethyl acetate was purchased from J. T. Baker (Deventer, Holland).

Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare buffers for CE. Other chemicals were obtained from Fluka (Buchs, Switzerland). All chemicals were of analytical reagent grade.

Apparatus and Conditions

CE was carried out with a laboratory-built system and with a commercial instrument. For the laboratory-built CE system, a Spellman power supply (Model RM15P10KD) was used (Plainview, New York, USA). A fused silica capillary of 50 cm effective length and 50 μm I. D. (Polymicro Technologies, Phoenix, AZ, USA) was used as the separation column. The peaks were detected by a Micro-UVis 20 detector (Carlo Erba, Milan, Italy) with wavelength set at 210 nm. The window for the on-column detection cell was made by removing a small section of the polyimide coating on the fused silica capillary. Data processing was performed with a Shimadzu (Kyoto, Japan) Chromatopac C-R6A integrator. Samples were injected into the capillary by gravity feed with an injection time of 5 seconds and an injection height of 10 cm. To obtain more reproducible data in the analysis of the real samples, the HP^{3D} CE system (Hewlett Packard, Palo Alto, CA, USA) was used for quantitative analysis. The same capillary was used in both systems. With the commercial instrument, samples were injected into the capillary by pressure (30 mbar, 20 seconds). The buffer solutions were sodium tetraborate - sodium dihydrogenphosphate solutions, modified by the appropriate cyclodextrin or cyclodextrins. Other conditions are described, where necessary, in the text.

Sample Preparation

Fifteen auxins were dissolved and diluted with methanol to obtain the final concentration of 27 μg/mL. The extraction procedure was modified from that described in a previous study.[12] Each sample of 30 g, fresh weight, was extracted with 80% methanol using a high speed blender maintained at 5°C. The extract was then filtered and the residue further extracted with cold methanol. 250 mL of cinnamic acid solution (200 μg/mL) was added as internal standard for quantification. 125 mL of IAA standard solution (200 μg/mL) was also added when recovery was determined. Methanol was removed from the pooled extract by evaporation, under reduced pressure, in a rotary evaporator. The aqueous concentrate was centrifuged for 10 min at 50,000 g. The supernatant was adjusted to pH 2.5 with 1 M HCl and partitioned 5 times with ethyl acetate. After adding molecular sieves (Type 4A) (Merck, Frankfurt, Germany), the acidic ether phases were kept overnight, then filtered and evaporated to dryness. The residues finally were dissolved in methanol in a 5 mL volumetric flask. The concentration of cinnamic acid in this methanolic solution was 10 μg/mL, and the concentration of the IAA standard was 5 μg/mL, when it was added to check the recovery.

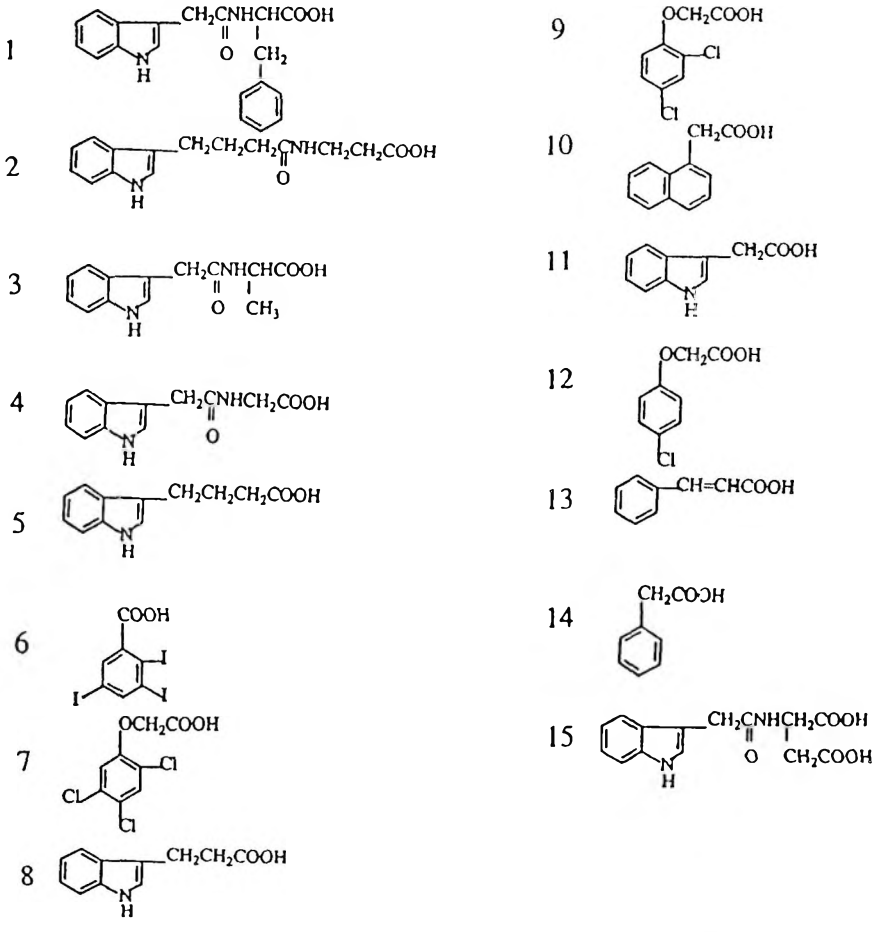

Figure 1. Structures of auxins. 1: Indole-3-acetyl-L-phenylalanine (IAPhe); 2: Indole-3-butyl-β-alanine (IBAla); 3: Indole-3-acetyl-L-alanine (IAAla); 4: Indole-3-acetylglycine (IAGly); 5: Indole-3-butyric acid (IBA); 6: 2,3,5-Triiodobenzoic acid (TIBA); 7: 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T); 8: Indole-3-propionic acid (IPA); 9: 2,4-Dichlorophenoxyacetic acid (2,4-D); 10: α-Naphthaleneacetic acid (NAA); 11: Indole-3-acetic acid (IAA); 12: p-Chlorophenoxyacetic acid (CPA); 13: t-Cinnamic acid (CA); 14: Phenylacetic acid (PAA); 15: Indole-acetyl-aspartic acid (IAAsp).

All the samples were stored at -4°C, and were passed through a Puradisc™ 25 TF filter (Whatman, UK) before CE analysis.

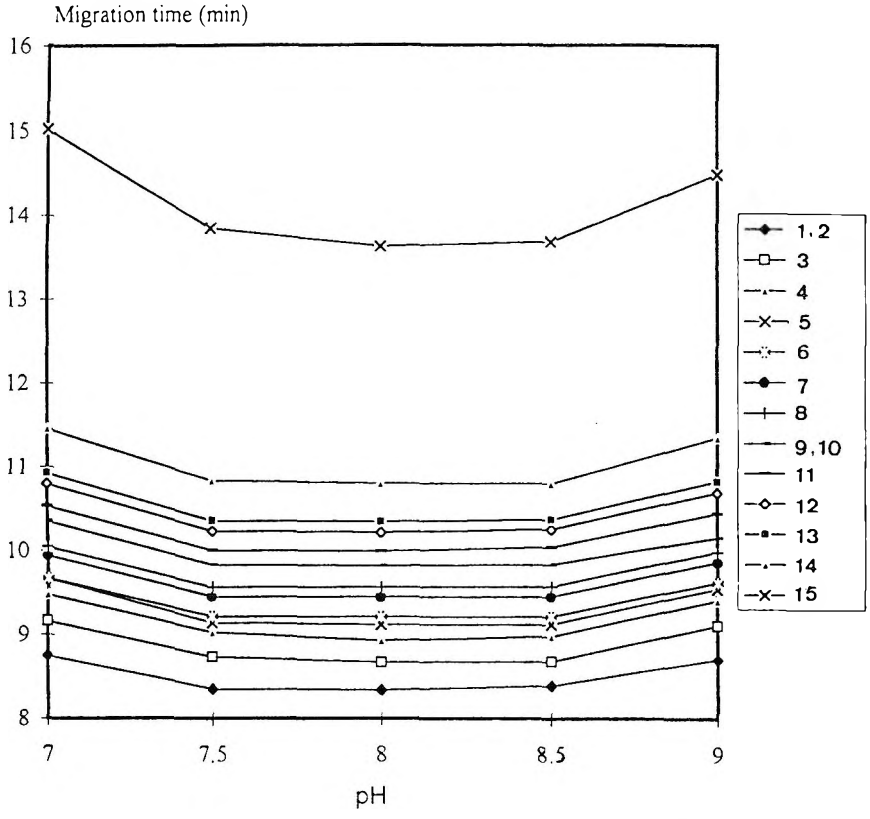

Figure 2. Influence of pH of buffer on migration times of auxins in CE system. Conditions: 25 mM borate-50 mM phosphate buffer; voltage, 15 KV; current, 23; UV (210 nm). Numbers as in Fig. 1.

Solution for Linearity Response

Seven solutions of different concentrations of IAA, which ranged from 0.4 to 6 μg/mL, were prepared from original compounds by dissolving and diluting with methanol. For each sample solution, a known amount of cinnamic acid was added at the concentration of 10 μg/mL.

RESULTS AND DISCUSSION

The names, abbreviations and structures of the auxins used in this paper are

listed in Figure 1. Among these compounds, eight are indole auxins, including IAA, IBA, IPA, and their amino acid conjugates, while others contain phenol groups. Therefore, all of them were amenable to detection in the UV range. All the compounds have low ionization constants (pK< 7) and, therefore, in high pH buffer solutions, they would exist as anionic compounds and are expected to be more easily separated by CE .

Effect of pH

Sodium borate and sodium dihydrohydrogen phosphate solution were used to prepare buffer solutions of pH 7 to 9. The effects of pH on migration times and resolution of auxins are shown in Figure 2 and Table 1, respectively. It was noted that, at pH >7, most of the auxins could be resolved using the borate-phosphate buffer except for two pairs of peaks, i.e. IAPhe and IBAla, and 2,4-D and NAA. However, increasing pH resulted in little change in the migration times and migration orders of the auxins.

Satisfractory separation was not obtained by varying pH alone. Nevertheless, slightly better separation and faster migration were observed in the pH range of 8.0-8.5. Therefore pH 8.4 was chosen to be the buffer pH in subsequent experiments.

The electrophoregram of auxins in 25 mM borate - 50 mM phosphate system at pH 8.4 is shown in Fig. 3. The migration behavior of the auxins could be attributed to differences in the electrophoretic flows of the anionic compounds, which would be in opposite direction to that of the electroosmotic flow, i.e. the ones with higher negative charges migrated slower.[13] The negative charges of the anionic compounds could be explained by inductive effects. IAAsp with two carboxyl groups possesses a higher negative charge than others, and migrated last.

The amino groups in other indole conjugates reduce their negative charges, and these compounds migrated faster than their indole acids. Among the indole acids, the migration order was based on the chain length of the side chains, i.e. less negative charge with longer carbon chains.

Among the conjugates, the migration times increased with the decrease of the isoelectric points of their amino acid groups.[14] As for the series of phenoxyacetic acids, their migration times decreased with the increase of the number of chloro substituents. TIBA, with iodine substituents, migrated faster than 2,4,5-T with chloro substituents. With conjugation of the double bond, cinnamic acid formed stable anions and migrated slower.

Table 1

Effect of pH on Resolution

Peak No.	pH				
	7	7.5	8	8.5	9
1,2	0	0	0	0	0
2,3	3.64	3.59	3.29	2.68	3.32
3,4	3.01	3.08	2.73	2.64	2.79
4,5	1.30	1.15	1.08	1.75	1.21
5,6	0.00	0.74	0.71	0.82	0.65
6,7	1.79	2.21	2.03	1.88	2.00
7,8	0.84	0.98	0.93	0.98	0.96
8,9	2.34	2.25	2.00	2.05	2.13
9,10	0.00	0.00	0.00	0.00	0.00
10,11	1.31	1.42	1.25	1.37	1.41
11,12	2.00	1.92	1.87	1.76	1.85
12,13	0.97	1.04	1.04	0.96	0.92
13,14	3.81	3.65	3.31	3.54	3.52
14,15	17.79	19.72	18.25	16.95	18.47

Effect of Cyclodextrins

Cylodextrins have been used extensively as modifiers in CE.[15-17] Enhancement of selectivity is attributable to differences in the stability of the cyclodextrin inclusion compounds, which could be influenced by many factors, such as hydrogen bonding, hydrophobic interaction, solvation power and the ability of the molecule to fill the cavity of the cyclodextrin.[13] In Fig. 4, the electrophoregramsobtained by adding 10 mM each of α-, β- and γ-cyclodextrins into the borate-phosphatebuffer at pH 8.4 are shown.

It can be observed that the migration order and resolution for the auxins changed significantly when different cyclodextrins were used. It was, therefore, expected that, by varying the type and concentration of the cyclodextrins used as modifier, it might be possible to obtain satisfactory separation of the auxins.

Subsequently, the effects of varying the concentrations of α-, β- and γ-cyclodextrins on the migration times of auxins were investigated. The results obtained confirmed that the migration times of each auxins differed significantly when different cyclodextrins were used. However, in the cases of α- and β-cyclodextrins, the migration times remained almost constant when different concentrationsof the cyclodextrinswere used.

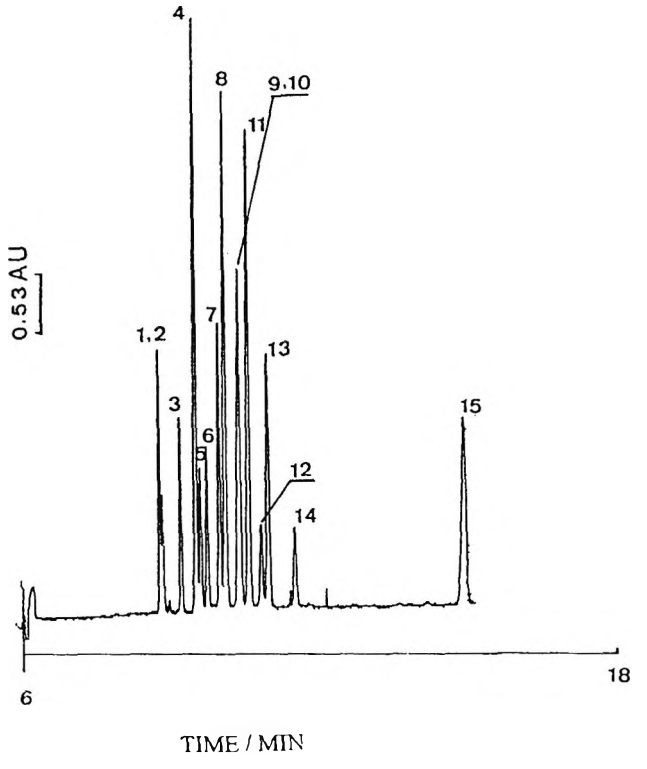

Figure 3. Capillary electropherogram of fifteen auxins without cyclodextrins. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

For γ-cyclodextrin, the migration times were found to increase when higher concentrations of the cyclodextrins were employed. Among the three cyclodextrins, α- and γ-cyclodextrins provided better resolution than β-cyclodextrin. Since β-cyclodextrin is relatively less soluble in aqueous solutions than the other two cyclodextrins,[18] it is possible that the inclusion complexes between the auxins and β-cyclodextrin were not as stable as those for the other two cyclodextrins and, thus, the effect of β-cyclodextrin on migration behaviour was less significant. In Figures 5 and 6, the effects of different concentrations of α- and γ-cyclodextrins on the migration times are shown. In Tables 2 and 3, the resolutions between adjacent pairs of peaks, determined from the migration time data for the two cyclodextrins, are given. For α-cyclodextrin, it was noted that relatively better resolutions between adjacent pairs of peaks observed at

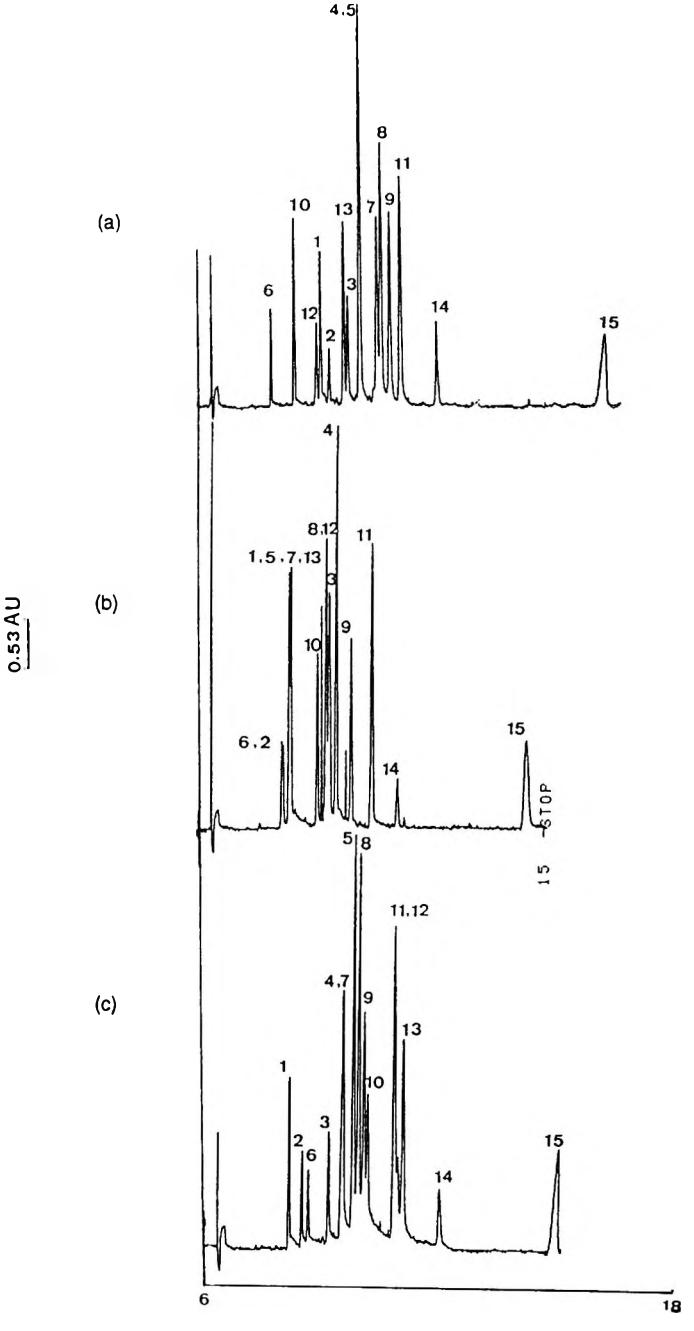

Figure 4. Capillary electropherograms of fifteen auxins using cyclodextrins as modifiers. a) with 10 mM α-CD; b) with 10 mM β-CD; c) with 10 mM γ-CD: Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

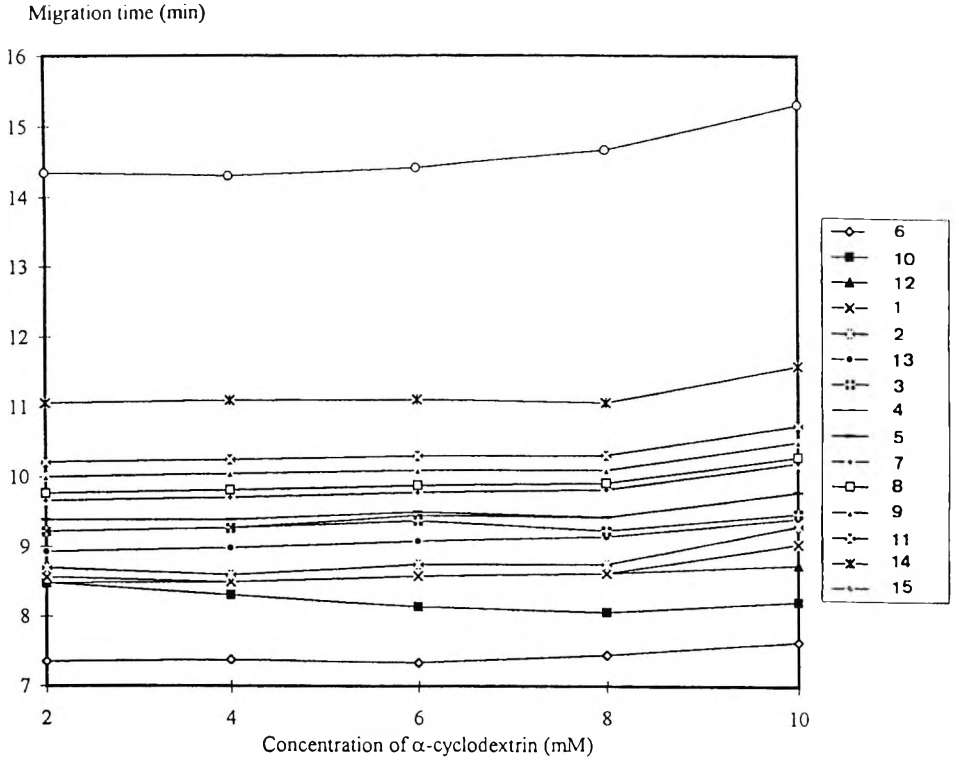

Figure 5. Influence of α-CD concentration on migration times of fifteen auxins in CE system. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

around 6 mM of α-cyclodextrin whereas, for γ-cyclodextrin, a concentration of around 2 mM was found to provide better separation of the auxins. However, it was found that, when each of the cyclodextrins was used individually, satisfactory separation of the fifteen auxins could not be obtained.

In view of the significant differences in migration times when different cyclodextrins were used, modifier systems consisting of mixtures of the cyclodextrins were considered as potentially more capable of enhancing selectivity in the separation of the auxins. Borate-phosphate buffer, modified with different mixtures of α- and γ-cyclodextrins were thus evaluated for the separation of auxins by CE. Satisfactory separation of all the fifteen peaks could be obtained using buffers containing 5.8 mM of α-cyclodextrin and 1.8 mM of γ-cyclodextrin.

Table 2

Effect of α-CD Concentration (mM) on Resolution

Peak No.	Concentration of α-CD				
	2	4	6	8	10
6,10	12.50	11.85	8.31	8.54	7.96
10,12	0.00	2.09	4.65	6.69	6.27
12,1	0.96	0.00	0.00	0.00	1.50
1,2	1.51	1.11	1.12	1.49	1.89
2,13	2.47	3.63	2.21	3.86	3.55
13,3	2.98	2.50	2.31	0.81	0.72
3,4	0.00	0.00	0.86	1.59	2.75
4,5	1.38	1.08	0.35	0.00	0.00
5,7	2.06	2.23	2.95	2.95	3.27
7,8	0.98	0.76	0.77	0.74	0.69
8,9	1.59	2.03	1.74	1.65	1.84
9,11	1.39	1.65	1.59	1.82	1.81
11,14	6.70	6.78	5.95	6.06	6.46
14,15	18.37	16.70	14.88	10.11	14.56

Other modifiers such as bile salts and methanols were also investigated. However, none of these modifiers provided better separation of the auxins than that obtained with the above mixture of α- and γ-cyclodextrins.

In summary, the optimum conditions for the separation of the auxins were determined as: applied voltage of 15 kV, a buffer solution containing 25 mM sodium tetraborate - 50 mM sodium dihydrogen phosphate (pH 8.4), 5.8 mM α-CD, and 1.8 mM γ-CD. A typical electropherogram of the auxins obtained using these conditions are shown in Fig. 7.

Determination of IAA in Vanda Miss Joaquim

Vanda Miss Joaquim (Vanda Hookeriana Rchb.f. x V. teres) is a terete, monopodial ground orchid. The inflorescence is axillary, usually develops at the second node from apex.[12] IAA percentage in the shoot apices of this plant was determined by CE using the optimum conditions. Cinnamic acid (No cinnamic acid was detected in this plant) was chosen to be the internal standard.

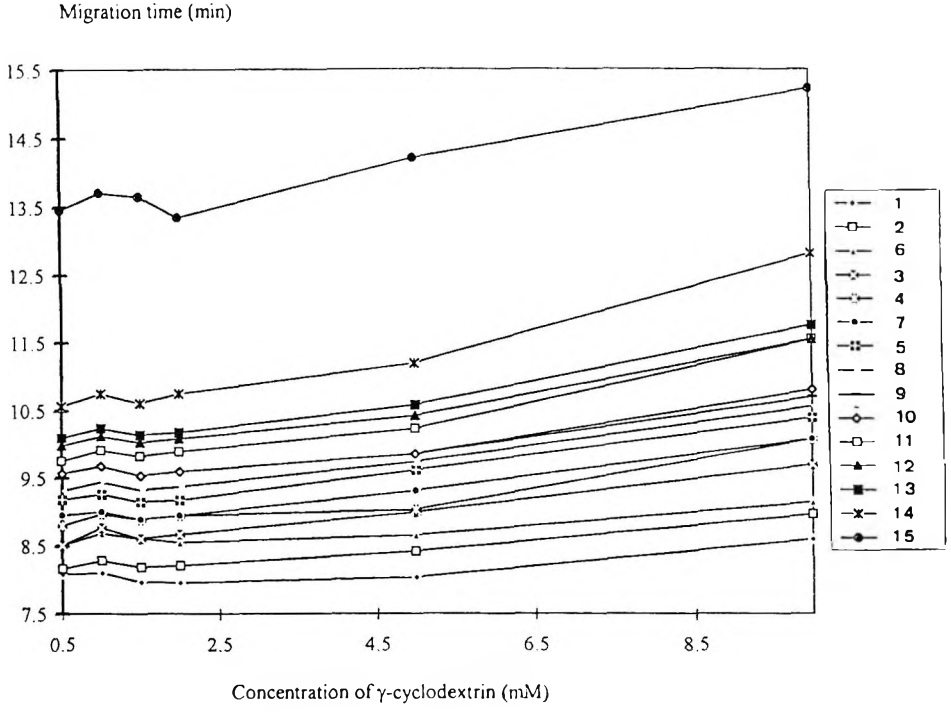

Figure 6. Influence of γ-CD concentration on migration times of fifteen auxins in CE system. Other separation conditions as in Fig. 2. Numbers as in Fig. 1.

Calibration graphs for IAA and cinnamic acid (peak-area ratio, y, vs concentration, x μg/mL) were constructed in the range 0.40-6.00 μg/mL (The limit of detection of the CE system was 0.40 μg/mL for IAA). The regression equation of the plot and its correlation coefficients were determined as follows:

$$y = 0.140x - 0.066 \qquad r = 0.9934$$

The electrophoregram of the plant extract is illustrated in Figure 8. Using the photodiode array detector in the HP^{3D} CE system, IAA and cinnamic acid were identified by their migration time and UV spectra (shown in Fig. 9). By substituting the area ratios of these peaks for y in the above equations, the concentrations of IAA in the final extract solutions were obtained (see Table 4). The content of IAA in fresh weight samples was determined and the result is shown in Table 4. In addition, the recovery of IAA, determined using the procedure described in the sample preparation section, is also listed in this table.

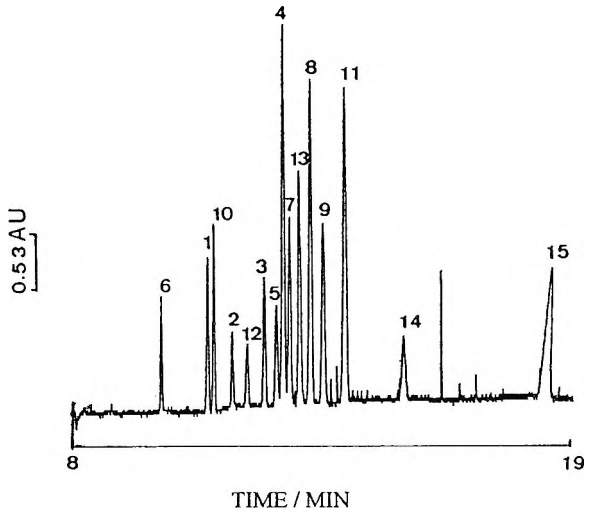

Figure 7. Capillary electropherograms of fifteen auxins obtained using the optimum conditions. Conditions: Buffer, 25 mM sodium tetraborate-50 mM sodium dihydrogenphosphate (pH 8.4), 5.8 mM α-CD and 1.8 mM γ-CD; UV detection at 210 nm; voltage, 15 KV; temperature, ambient. Numbers as in Fig. 1.

Table 3

Effect of γ-CD Concentration (mM) on Resolution

Peak No.	Concentration of γ-CD					
	0.5	**1**	**1.5**	**2**	**5**	**10**
1,2	1.00	2.10	2.67	3.16	3.17	3.26
2,6	3.33	4.28	4.08	3.79	0.00	1.49
6,3	0.00	0.79	0.00	1.20	3.79	4.52
3,4	2.13	1.94	2.38	2.64	2.45	2.88
4,7	1.34	0.33	0.00	0.00	0.00	0.00
7,5	2.23	2.38	2.19	2.03	2.13	2.15
5,8	1.17	1.61	1.55	1.82	1.12	1.06
8,9	2.22	2.06	1.75	1.97	1.45	0.92
9,10	0.00	0.00	0.00	0.00	0.34	0.61
10,11	1.58	2.04	2.48	2.45	2.98	3.96
11,12	1.87	1.68	1.62	1.67	0.00	0.00
12,13	1.02	0.95	0.84	1.03	1.24	1.11
13,14	3.75	4.18	5.49	4.32	6.02	6.13
14,15	15.27	16.44	19.99	15.77	15.89	17.82

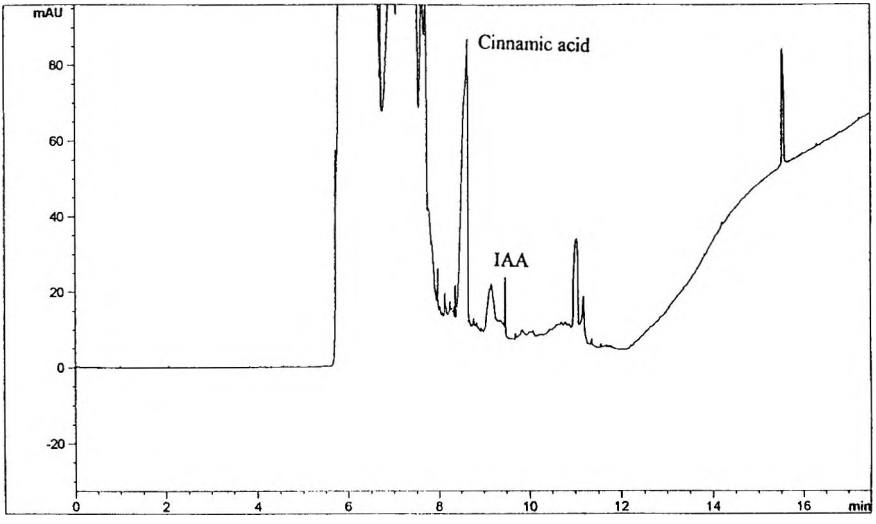

Figure 8. Capillary electropherogram of the extract of Vanda Miss Joaquim. Separation conditions as in Fig.7.

Table 4

Content of IAA in Real Sample (n = 3)

Sample	Area Ratio (IAA/Cinnamic Acid)	Concentration (μg/mL)	IAA Content (μg/g)	Recovery
Extract	0.1443	0.561	0.093	
Extract + 5 μg/mL IAA	0.7184	4.673	0.779	82.40%

IAA content in this kind of plant was previously determined by bioassay.[12] However, only a crude range of IAA concentration was obtained. The CE method described in this work is relatively more rapid and precise and, therefore, should be useful for quantitative analysis of auxins in plant samples.

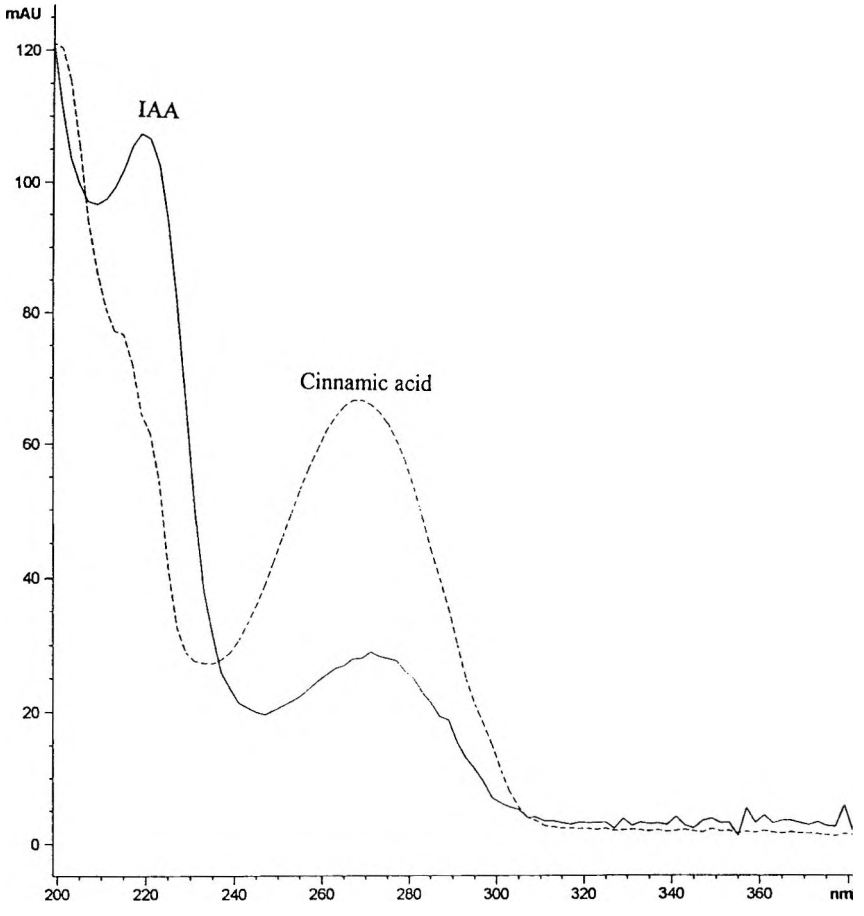

Figure 9. UV spectra of IAA and cinnamic acid in CE system. Wavelength: 190-380 nm.

CONCLUSION

In this study, a method for the separation and determination of auxins by capillary electrophoresis was developed. Optimum conditions for the separation of auxins were obtained.

A simple and reliable analytical procedure was described, which was demonstrated successfully as a suitable method for the determination of IAA in a real plant extract sample.

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REFERENCES

1. P. J. Davies, **Plant Hormones and Their Role in Plant Growth and Development**, Martinus Nijhoff Publishers, Dordrecht, 1987, Ch. 1, p. 4.

2. J. Ludwig-Muller, S. Sass, E. G. Sutter, M. Wodner, E. Epstein, Plant Growth Regulation **13**, 179-187 (1993).

3. J. Badenoch-Jones, R. E. Summons, B. G. Rolfer, D. S. Letham, J. Plant Growth Regul. **3**, 23-29 (1984).

4. F. Wigthman, D. L. Lighty, Physiol. Plant, **55**, 17-24 (1982).

5. T. Shen, J. Y. Wang, **Biochemistry**, Education Publishers, Beijing, 1989, Ch. 8, p. 466.

6. G. Sandberg, A. Crozier, A. Ernsten, in **Principles and Practice of Plant Hormone Analysis**, L. Rivier, A. Crozier, editors, Academic Press Inc., London, 1987, Ch. 4, p. 169.

7. P. Hernandez, F. Galan, O. Nieto, L. Hernandez, Electroanalysis, **6**, 577-583 (1994).

8. S. K. Yeo, H. K. Lee, S. F. Y. Li, J. Chromatogr., **594**, 335-340 (1992).

9. J. D. Cohen, Plant Physiol., **70**, 749-753 (1982).

10. E. A. Schneider, C. W. Kazakoff, F. Wightman, Planta, **165**, 232-241 (1985).

11. F. Sitbon, A. Ostin, B. Sundberg, O. Olsson, G. Sundberg, Plant Physiol., **101**, 313-320 (1993).

12. C. J. Goh, H. Y. Wan, **Plant Growth Substances, 1973**, Y. Sumiki, editor, Hirokawa Publishing Co., Tokyo, 1974, Ch. 7., p. 945.

13. S. F. Y. Li, **Capillary Electrophoresis: Principles, Practice and Application**, Elsevier, Amsterdam-London-NewYork-Tokyo, Ch. 1, p. 12.

14. T. Shen, J. Y. Wang, **Biochemistry**, Education Publishers, Beiing, 1987, Ch. 3, p. 78.

15. J. Liu, K. A. Cobb, M. Novotny, J. Chromatogr., **519**, 189-197 (1990).

16. Y. Y. Rawjee, D. U. Staerk, G. Vigh, J.Chromatogr., **635**, 291-306 (1993).

17. D. Belder, G. Schomburg, J. Chromatogr., **666**, 351-365 (1994).

18. J. Snopek, I. Jelinek, E. Smolkova-Keulemansova, J. Chromatogr., **452**, 571-590 (1988).

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MICELLAR HPLC: INVESTIGATION OF THE RETENTION OF POSITIVELY CHARGED PEPTIDES USING CATIONIC MICELLAR MOBILE PHASES

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ABSTRACT

Many new and complex molecules are being investigated as potential drug candidates. Conventional analytical methodologies may not be suitable for determining the purity, identity, and degradation of these complex molecules. Therefore, new analytical techniques must be developed that address and overcome these problems. Micellar liquid chromatography is such a technique.

Micellar liquid chromatography (MLC) is a technique where a micellar agent is added to a mobile phase that contains a buffer and a small amount of organic modifier. Several advantages are apparent with MLC when compared to reversed-phase liquid chromatography. MLC uses a much lower amount of organic modifier and is therefore less toxic, MLC does not denature peptides and proteins as does RPLC, and gradient MLC is done without the need for long column re-equilibration. In this study, various mobile phase variables were studied to determine the effect that each had on peptide retention.

The variables studied include: the concentration of micellar agent, mobile phase ionic strength, concentration of buffer, concentration of organic modifier and mobile phase pH. The results that were obtained are discussed.

INTRODUCTION

The separation and quantitation of complex molecules has become a very important part of analytical chemistry. Many of these complex molecules are being synthesized as potential new drug candidates. Several different research techniques are being used to identify and develop new drug candidates, including: biotechnology, molecular modeling, and natural product discovery. In many of these cases, the drug candidates are large, complex molecules that are difficult to assay for purity using conventional analytical methodologies. Therefore, new analytical strategies and techniques are required to determine the purity of the drug substance, to identify any impurities or degradation products, and to fully characterize the new chemical entity.

Several separation schemes have been shown to be useful for separating complex molecules and include: high performance liquid chromatography (HPLC), and capillary zone electrophoresis (CE) and, to a much lesser extent, gas chromatography, and supercritical fluid chromatography. Problems have been associated with these separation techniques and, although each holds promise, none have been found to be acceptable for the routine analysis for all types of complex molecules.

Capillary zone electrophoresis (CE) has shown great promise for the separation of various types and sizes of molecules in the biological sciences.[1] In the area of peptide and protein separations, CE can provide analytical chemistry with some very interesting and potentially outstanding separations. These types of analytes can be separated based on charge differences at a given pH.

A drawback to this strategy is that proteins may not be stable in low pH buffers. Another problem is that increased temperatures may result from Joule heating which can denature proteins and give inaccurate purity information.[2] A reliable, consistent injection system is also required before CE will become as routinely used as HPLC. This has been a major stumbling block for the use of CE in QA/QC labs, as has the cost of the instrumentation. Most QA/QC labs already have HPLC instrumentation in place and the addition of a CE unit may be cost prohibitive.

Ion exchange chromatography has been successfully employed for the separation of peptides.[3,4] Ion exchange columns, however, are much less efficient than reversed-phase columns and may not provide the kind of resolution that would be required to separate similar peptides.

An alternative to these analytical techniques would be micellar HPLC (MLC). The advantages of using MLC in place of reversed-phase or ion exchange chromatography are extensive when protein separations are being investigated. Reversed-phase HPLC (RPLC) requires the use of high organic modifier concentrations. This may denature the peptides and prevent complete resolution of the analyte peak of interest and possible impurities. Silica based columns are typically used for these separation and are limited to a pH range of 2.0-7.0. In some cases, a higher pH would provide a better separation for the analyte of interest. A polymeric-based column may be used to overcome the pH limitation of the silica-based columns; however, the efficiency of polymer columns is significantly less than that of silica-based columns.

Several interesting separations have been accomplished using MLC. Cline Love and co-workers[5-7] reported the direct injection of serum and urine into a reversed-phase column with no protein precipitation or pressure build-up problems. This method was used for therapeutic drug monitoring without the requirement of sample cleanup prior to injection. MLC has been shown to be useful for the separation of amino acids and peptides,[8] and proteins.[9] One study found that small changes in the concentration of surfactant produced tremendous changes in the retention of different proteins.[9] Other protein separations that are being pursued include one in which the biological activity of the protein is maintained.[10,11]

The purpose of this research was to determine the effects that each mobile phase variable had on peptide retention and resolution using micellar liquid chromatography. The mobile phase variables that were studied include: the concentration of micellar agent, mobile phase ionic strength, concentration of buffer, concentration of organic modifier and mobile phase pH. The results from these studies are discussed.

EXPERIMENTAL

Reagents and Instrumentation

All of the peptides used in this study were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile was obtained from Burdick and Jackson (Muskegon, MI, USA). Phosphoric acid, sodium monobasic

phosphate, and sodium hydroxide were obtained from Mallinckrodt (Paris, KY, USA). Hexadecyltrimethylammonium bromide was purchased from J. T. Baker (Phillipsburg, NJ, USA). HPLC grade water was obtained by passing de-ionized water through a Nanopure II water purification system (Barnstead, Dubuque, IA, USA).

The instrumentation consisted of a Spectra Physics P4000 Quaternary Pump, Spectra Physics AS3000 Autosampler, Spectra Physics Spectra UV1000 detector, and Spectra Physics ISM100 (Fremont, CA, U.S.A.). The Zorbax Rx C-18 column (4.6 x 250 mm, 5 μm) was purchased from Mac-Mod Analytical (Chadds Ford, PA, USA).

Procedures

The peptide samples were prepared at a concentration of 1 mg/g of HPLC-grade water. A flowrate of 1.0 mL/min. was used for all separations along with UV detection at 220 nm, an injection volume of 20 μL and a column temperature of 30 ^{o}C.

RESULTS AND DISCUSSION

Micellar liquid chromatography (MLC) provides a very convenient and reproducible route for separating peptides without the problems associated with CE, RPLC and ion exchange chromatography. MLC mobile phases consist of a surfactant, a buffer, and a low concentration of organic modifier. High column efficiencies are achieved since silica-based reversed-phase columns are used. A major advantage to MLC is that the mobile phases contain a much lower concentration of organic modifier than a reversed phase system and are therefore less toxic.

The surfactants used in MLC consist of two portions that contain distinctly different properties: a polar head group and a hydrocarbon tail. These properties allow the surfactant to adsorb at interfaces (stationary phase) where both the hydrophobic and hydrophilic character can be satisfied. The formation of micelles is the result of opposing forces– hydrophilic and hydrophobic. When the critical micelle concentration is achieved, the surfactant molecules arrange in such a way that the hydrophobic tails are oriented toward the center of the aggregate and the polar heads point outward.[12] The repulsion between the polar head groups is the controlling force that determines the size and shape of the micelles.

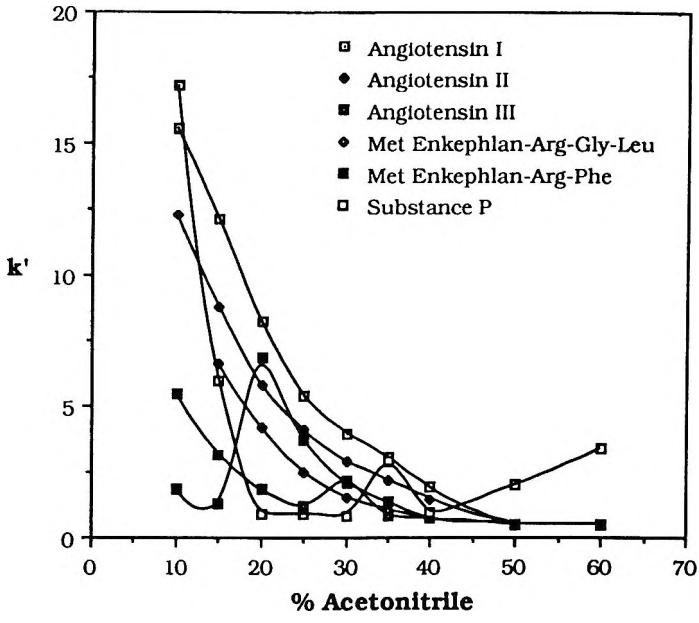

Figure 1. Effect of Organic Modifier Concentration on Peptide Retention. Mobile phase: 5 mM $HDTMA^+Br^-$, 15 mM H_3PO_4, pH 7.0, CH_3CN.

The separation mechanism in MLC is similar to RPLC in that the primary equilibrium of the analyte is between the mobile phase and the stationary phase. In MLC a secondary equilibrium is also involved in the separation. This equilibrium is the partitioning of the analyte between the mobile phase and the micelles.[12,13]

Various mobile phase parameters will have an effect on the retention and separation of organic analytes such as peptides. The parameters that were studied include: concentration of surfactant, buffer concentration, mobile phase ionic strength, concentration of organic modifier and mobile phase pH.

Effect of Organic Modifier Concentration

The amount of organic modifier present in the mobile phase will have an effect on analyte retention. Khaledi and co-workers[14] have shown that elution strength increased with an increase in the organic solvent concentration. A corresponding enhancement in the separation selectivity was also observed. The selectivity enhancement was found to occur systematically and was observed for

a large number of ionic and nonionic compounds with different functional groups, and also for two different surfactants, one anionic and one cationic. The selectivity enhancement was credited to competing partitioning equilibria in micellar HPLC systems and/or to the characteristics of micelles to compartmentalize solutes and organic solvents.[14]

Some concern has been expressed that micellar mobile phases would act like a hydro-organic system at higher concentrations of organic modifier. This, however, was shown not to be the case. It has been demonstrated that a micellar eluent that contains up to 20% isopropanol does not change to a hydro-organic system.[8] The addition of an organic modifier actually enhances the solvent strength and selectivity for some ionic and nonionic analytes. Retention characteristics for a solvent-water-micellar system were also found to be similar to a purely aqueous micellar eluent.[15,16] It was concluded, from these studies, that the micelle influences the role of an organic modifier in the mobile phase.

Figure 1 shows the effect on peptide retention when the amount of acetonitrile added to the mobile phase was changed. When the concentration of acetonitrile was less than 10%, retention of the peptides was extremely high. It was found that the retention of the peptides generally decreased with increasing concentrations of acetonitrile. One peptide, substance P, was found to increase in retention. This increase in retention is most likely attributable to changes in interactions between the micelles and the solvent.[8] The organic modifier concentration must be chosen such that the peptides are resolved yet retention is not excessive.

Effect of Micellar Concentration

When the concentration of a micellar agent was increased in the mobile phase, a corresponding decrease in analyte retention was usually observed.[17] The rate at which the retention of the analyte changes varies with the charge and hydrophobicity of solutes as well as the length of the alkyl chain, charge and concentration of the micelles.[18] A study done by Bailey and Cassidy[19] showed that the efficiency of the micellar system improved for hydrophobic analytes but not for polar analytes as the micellar concentration was increased.

Figure 2 shows how the concentration of hexadecyltrimethylammonium bromide (HDTMABr) influenced peptide retention. As the mobile phase concentration of HDTMABr was increased, peptide retention was found to decrease. This would be expected since at low concentrations of micellar agent, the chromatographic system resembles conventional reversed-phase LC. As the concentration of micellar agent is increased, the number of micelles in the system increases and binding between the analyte and the micelle increases.[20]

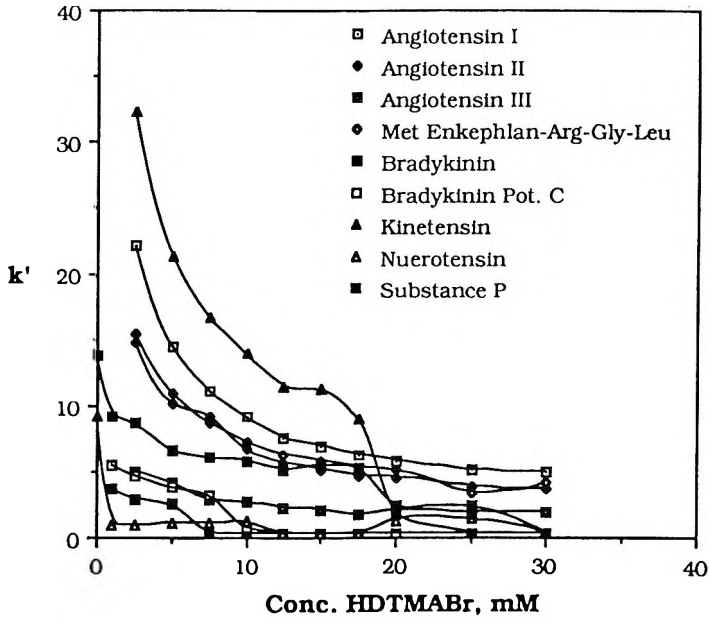

Figure 2. Effect of HDTMABr Concentration on Peptide Retention. Mobile phase: $HDTMA^{+}Br^{-}$, 15 mM H_3PO_4, pH 7.0, 20% CH_3CN.

Changes in elution order were observed and are due to differences in the binding constants of the micelle and the analyte. Selectivity between analytes may change due to the contribution of electrostatic and hydrophobic interactions, which is dependent on the structure of the compound. Selectivity changes have also been observed for diverse pairs of zwitterionic amino acids and peptides with changing micellar concentrations.[21]

The solvent strength of the mobile phase increased at higher concentrations of micellar agent. However, this increase has a negative effect on the efficiency of the chromatographic system.[20] Therefore, care must be taken when choosing an appropriate amount of micellar agent for a desired separation.

In the case of the peptides in this study, a concentration of 5.0 mM HDTMABr appears to provide the best compromise between retention, selectivity, and efficiency.

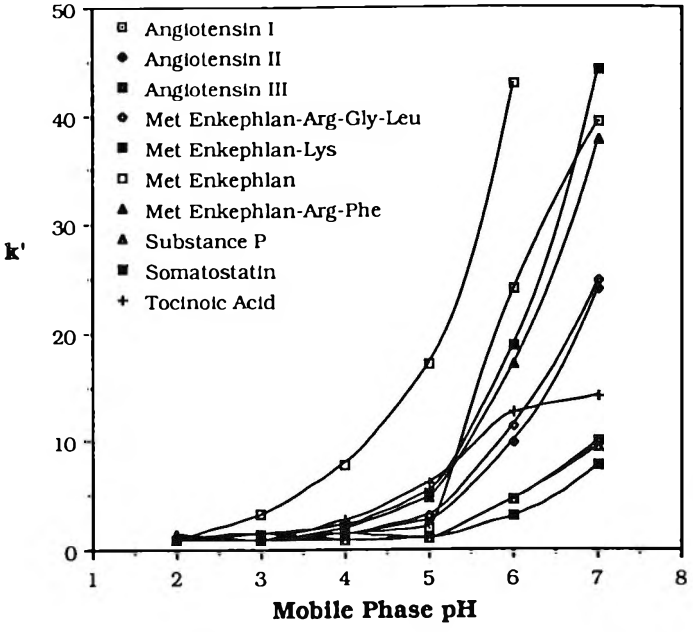

Figure 3. Effect of Mobile Phase pH on Peptide Retention. Mobile phase: 5 mM $HDTMA^{+}Br^{-}$, 15 mM H_3PO_4, 20% CH_3CN.

Effect of Mobile Phase pH

The micellar mobile phase pH will have a dramatic effect on the retention of weak organic acids and bases. Partition coefficients for the micelle-analyte interactions are different for the associated and unassociated forms.

Several studies have shown that small changes in the mobile phase pH will have an effect on retention especially when the mobile phase pH is close to the analyte's pK_a value.[22-24]

Adsorption of anionic surfactant monomers on the surface of a C_{18} stationary phase cause protonated organic bases to be retained for a longer period of time than the neutral free-base form due to electrostatic attraction.[20] Research has also shown that the dependence of k' on pH at a constant concentration of micellar agent is sigmoidal if there is no electrostatic repulsion between any of the acid-base forms and surfactant molecules.[25]

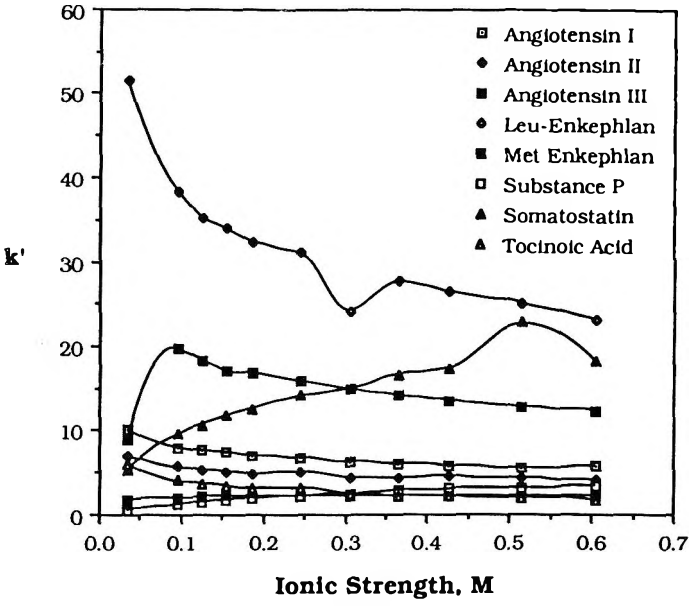

Figure 4. Effect of Mobile Phase Ionic Strength on Peptide Retention. Mobile phase: 5 mM HDTMA $^{+}Br^{-}$, H_3PO_4, pH 7.0, 20% CH_3CN.

The retention of the peptides in this study were found to decrease when the mobile phase pH was lowered (Figure 3). It is interesting to note that this is the opposite of what would be expected if the micellar agent was anionic (sodium dodecyl sulfate) rather than the cationic HDTMABr. Rodgers and Khaledi[26] showed that amino acids increased in retention as the mobile phase pH was lowered from 5.5 to 2.5 when an anionic surfactant, sodium dodecyl sulfate (SDS), was used. This was attributable to electrostatic repulsion between the solute and surfactant at zwitterionic conditions. It was also shown that the retention of amino acids, using nonionic micelles, pass through a retention minima at zwitterionic pH conditions. In both cases, analyte retention decreased with increasing anionic micelle concentration.

The cationic HDTMABr surfactant used in this study repels the positively charged peptides which leads to lower retention times. When the peptides are zwitterionic, interactions between the peptide and the cationic HDTMABr may take place thus leading to higher retentions. Therefore, for the peptides to be retained using a cationic surfactant, a higher mobile phase pH is required.

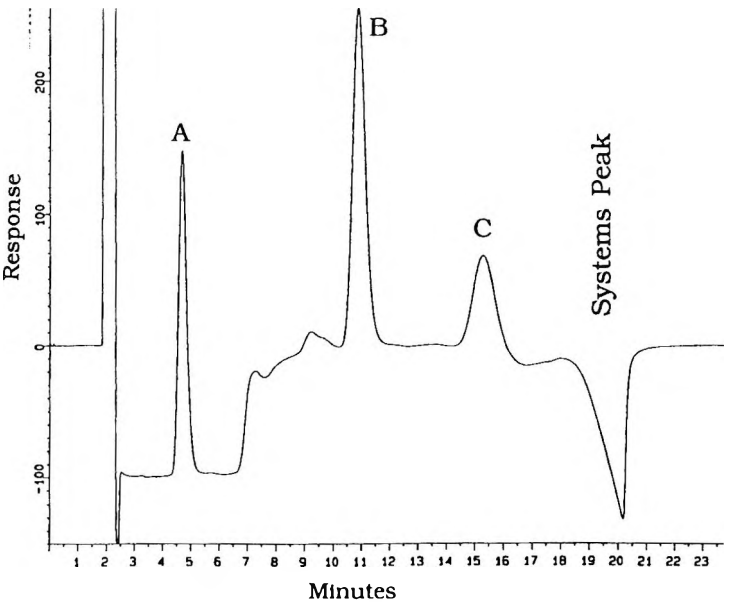

Figure 5. The separation of Angiotensin I, II and III by micellar liquid chromatography. Mobile phase: 5 mM HDTMA $^{+}Br^{-}$, 15 mM H_3PO_4, pH 7.0, 20% CH_3CN. A) Angiotensin III, B) Angiotensin II, C) Angiotensin I.

Effect of Mobile Phase Ionic Strength

In micellar liquid chromatography, electrostatic interactions are involved between a charged analyte and the micelle in the diffuse secondary layer while hydrophobic interactions take place in the hydrophobic inner portion of the micelle. Armstrong and Stine[27] have shown that the thickness of the double layer decreases with increasing ionic strength, which allows hydrophobic interactions to take place between the analyte and the micelle.

Anti-binding analytes (compounds that are strongly excluded or repelled from a micelle) have been found to have increased retention with higher ionic strength mobile phases.[20] For the transition from anti-binding to non-binding to binding to occur, the analyte ion must have enough hydrophobic character to associate with the non-polar portion of the micelle, overcoming electrostatic repulsion. Bromophenol blue has been shown to change from an anti-binding to a binding analyte with a corresponding increase in retention using an SDS mobile phase with 0.02 M NaCl added.[27]

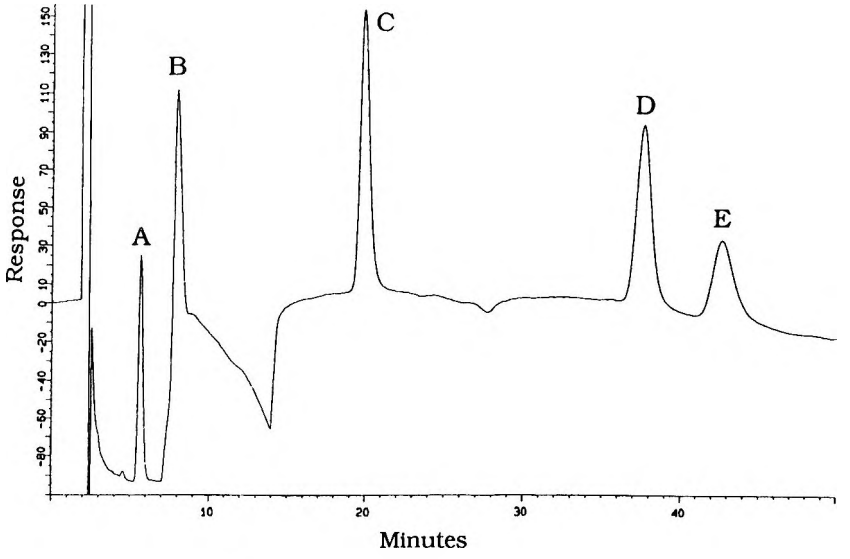

Figure 6. The separation of Enkephalins by micellar liquid chromatography. Mobile phase: 5 mM HDTMA$^+$Br$^-$, 15 mM H_3PO_4, pH 6.0, 20% CH_3CN. A) Met-Enkephalin-Arg-Gly-Leu, B) Met-Enkephalin-Arg-Phe, C) Derm-Enkephalin, D) Leu-Enkephalin, E) Met-Enkephalin.

Several changes were observed for the retention of the peptides when the mobile phase ionic strength was increased (Figure 4). Some of the peptides showed a reduction in retention, several did not show much change in retention and some showed a large increase in retention. The peptides that showed an increase in retention with increasing ionic strength (e.g., somatostatin) are changing from an anti-binding to a binding character, whereas the peptides that are decreasing in retention may be considered to have binding characteristics (e.g., Leu-enkephalin). The peptides that do not change in retention may be considered to have non-binding character. Interesting changes in selectivity and elution order can take place with different ionic strength mobile phases. However, for the separation of the different peptides to take place, lower ionic strength mobile phases are desirable.

Separations

The separation of a mixture of different peptides was accomplished. Figure 5 shows the separation of Angiotensin I, II and III while Figure 6 shows the separation for several Enkephalins. The Angiotensin samples were better

resolved at a mobile phase pH of 7.0 where the peptides were more highly retained, whereas the Enkephalins had a better separation at pH 6.0. At pH 7.0, some of the Enkephalins had extremely long retention times and were not observed after 90 minutes. Therefore, the pH of the mobile phase was lowered so that the Enkephalins would elute in a reasonable amount of time. Overall, the peptides were well resolved and the separations for the complex mixtures were acceptable.

CONCLUSIONS

The use of micellar mobile phases for the separation of peptides was studied. The retention of the different peptides were found to be affected by different mobile phase parameters. The various parameters were identified and studied. It was found that the use of a micellar mobile phase for the separation of short to medium chain peptides is possible. Further studies are ongoing to determine how other types of micellar agents will affect the retention and separation of peptides.

REFERENCES

1. B. L. Karger, A. S. Cohen, A. Guttman, J. Chromatogr., **492**, 585 (1989).

2. R. M. McCormick, Anal. Chem., **60**, 2322 (1988).

3. P. Mychack, J. R. Benson, LC-GC, **4**, 462 (1986).

4. R. A. Bradshaw, O. J. Bates, J. R. Benson, J. Chromatogr., **187**, 27 (1980).

5. F. J. DeLuccia, M. Arunyanart, L. J. Cline Love, Anal. Chem., **57**, 1564 (1985).

6. M. Arunyanart, L. J. Cline Love, J. Chromatogr., **342**, 293 (1985).

7. F. J. DeLuccia, M. Arunyanart, P. Yarmchuk, R. Weinberger, L. J. Cline Love, LC Magazine, **3**, 794 (1985).

8. A. S. Kord, M. G. Khaledi, J. Chromatogr., **631**, 125 (1993).

9. R. A. Barford, B. J. Sliwinski, Anal. Chem., **56**, 1554 (1984).

10. K. Ohbu, N. Jona, N. Miyajima, N. Mizushima, I. Kashiwa, Yakagaku, **29**, 866 (1980).

11. T. Asakura, K. Adachi, E. Schwartz, J. Biol. Chem., **253**, 6423 (1978).

12. D. W. Armstrong, Separation and Purification Methods, **14(2)**, 213 (1985).

13. J. G. Dorsey, M. G. Khaledi, J. S. Landy and J. L. Lin, J. Chromatogr., **316**, 183 (1984).

14. M. G. Khaledi, J. K. Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

15. M. G. Khaledi, E. Peuler, J. Ngeh-Ngwainbi, Anal. Chem., **59**, 2738 (1987).

16. M. G. Khaledi, Anal. Chem., **60**, 876 (1988).

17. D. W. Armstrong, G. Y. Stine, Anal. Chem., **55**, 2317 (1983).

18. A. S. Kord, M. G. Khaledi, Anal. Chem., **64**, 1894 (1992).

19. R. Bailey, R. M. Cassidy, Anal. Chem., **64**, 2277 (1992).

20. M. J. M. Hernandez, M. C. G. Alvarez-Coque, Analyst, **117**, 831 (1992).

21. M. G. Khaledi, J. K Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

22. F. Palmisano, A. Guerrieri, P. G. Zambonin, T. R. I. Cataldi, Anal. Chem., **61**, 946 (1989).

23. L. J. Cline Love, J. Fett, J. Pharm. Biomed. Anal., **9**, 323 (1991).

24. J. Haginaka, J. Wakai, H. Yasuda, J. Chromatogr., **488**, 341 (1989).

25. M. Arunyanart, L. J. Cline Love, Anal. Chem., **57**, 2837 (1985).

26. A. H. Rodgers, M. G. Khaledi, Anal. Chem., **66**, 327 (1994).

27. D. W. Armstrong, G. Y. Stine, J. Am. Chem. Soc., **105**, 6220 (1983).

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ISOLATION OF VARIOUS AZADIRACHTINS FROM NEEM OIL BY PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Isolation of various azadirachtins, ie., Azadirachtin A,B, D, H and I in pure form from neem oil by preparative high performance liquid chromatography procedure is described.

INTRODUCTION

In a recent publication in this journal we had reported the isolation of the major constituents of neem oil (oil from the seeds of *Azadirachta indica* A.Juss.),[1] like salannin, nimbin, deacetylnimbin, azadiradione and epoxyazadiradione by direct preparative high performance liquid chromatography using a reverse phase column. However, the first two peaks (peak 1 and peak 2 in ref[1]) in these preparative runs gave relatively small amounts of material and the compounds present were not isolated although some of them were identified and quantitated, such as azadirachtin A, B, D, H

and I by analytical HPLC. In a very recent publication[2] HPLC analysis of neem oil using a semi-preparative reverse phase column has been carried out and a few major triterpenoids like nimbandiol, nimbanol, salannin have been identified by HPLC-MS procedure and quantified by the mass intensities, but the compounds were not isolated.

Herein, we report the actual isolation of various individual azadirachtins present in neem oil by preparative HPLC procedure.

MATERIALS AND METHODS

Preparative High Performance Liquid Chromatography was carried out using a Shimadzu LC8A HPLC system linked to CR4A data processor and the peaks detected at 215 nm. Two Shimpack reverse phase (C_{18}) preparative columns (25 cmx50 mm i.d.) and (25 cm x 20 mm i.d.) were used for preparative runs and Phenomenex reverse phase column (C_{18}) (25 cm x 4.6 mm) was used for analysis.

Neem oil, (1 lit) obtained by using a cold mechanical expeller was partitioned between n-hexane and 90% methanol and the methanol extract was concentrated to dryness *in vaccuo* at 45° (62.8g). This was subjected to preparative HPLC for the isolation of major triterpenoids.

RESULTS AND DISCUSSION

For each preparative run 5 g of the above residue from methanol extract was dissolved in 20 mL of methanol, filtered through a Millipore filter (0.25μm) and then injected into the preparative column (20 cm x 50 mm i.d). The eluent flow rate was 30 mL/min throughout the run.

During the first sixty minutes 60:40 MeOH:H_2O was used and the more polar compounds like azadirachtins were eluted. From 60 minutes the eluent was changed to 70:30 MeOH:H_2O and atleast six major peaks eluted out. The identities of the compounds in these six peaks have already been described.[1]

We report here the actual isolation of various azadirachtins from the peak-1 (rt. 34.3 min.) and peak-2 (rt. 43.7 min.)[1] obtained during the first sixty minutes by subjecting them to further preparative high performance liquid chromatography.

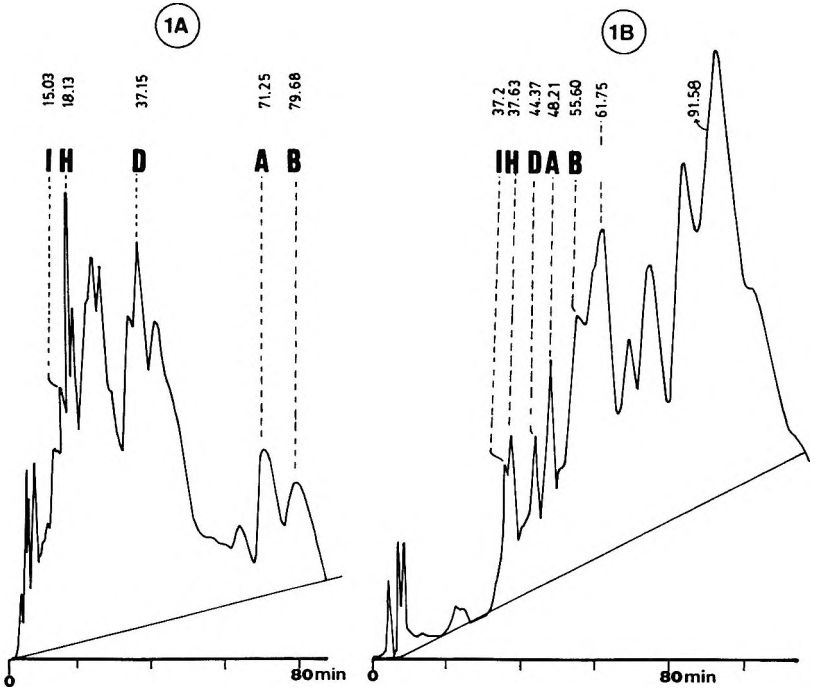

Figure. 1A Preparative High Performance Liquid Chromatogram of Peak-1
Figure. 1B Preparative High Performance Liquid Chromatogram of Peak-2

Peak-1 (3g, 500mg/4 mL MeOH for each run) was subjected to preparative high performance liquid chromatography using 25 cm x 20 mm i.d. column with 28:72 CH_3CN:H_2O as eluent at 12 mL/min flow rate (Fig. 1A). The individual peaks were collected and evaporated to yield various azadirachtins, the identity of these were established by analytical HPLC using 25 cm x 4.6 mm i.d. column with 15 : 35: 50 CH_3CN : MeOH : H_2O as eluent at 1mL/min flow rate and comparing the retention times with authentic azadirachtin samples.

Thus peaks with retention times 15.03 min. (132 mg), 18.13 min.(264 mg), 37.15 min.(310 mg), 71.25 min. (180 mg) and 79.68 min.(300 mg) were identified to be pure Azadirachtin I, Azadirachtin H, Azadirachtin D, Azadirachtin A and Azadirachtin B respectively (Table 1).

Table 1

Isolation of Various Azadirachtins from Peaks 1 and 2 in Neem Oil

Sl. No.	Compound	mg of Compound		Total	% Isolated from Neem Oil
		Peak 1	Peak 2		
1	Azadirachtin I	132	56	188	0.0188
2	Azadirachtin H	264	168	432	0.0432
3	Azadirachtin D	310	100	410	0.0410
4	Azadirachtin A	180	99	279	0.0279
5	Azadirachtin B	300	172	472	0.0472

Pk-2 (3.5 g , 500mg/4 mL MeOH for each run) was subjected to preparative high performance liquid chromatography using 25 cm x 20 mm i.d. column with 28:72 CH_3CN : H_2O as eluent and 15 mL/min flow rate (Fig. 1B). The individual peaks were collected and evaporated to yield various azadirachtins, the identities of these were done by analytical HPLC as indicated for peak 1.

Thus peak 2 yielded Azadirachtin I(56 mg), Azadirachtin H (168 mg), Azadirachtin D (100 mg), Azadirachtin A (99mg) and Azadirachtin B (172 mg) respectively (Table 1).

In peak-2 two more peaks with retention times of 61.75 min. (48 mg) and 91.58 min.(165 mg) were collected, but the identities of these compounds have yet to be established. The identity and purity of the individual azadirachtins were further confirmed by spectral data and comparison with standard samples by analytical HPLC.

It is evident that peak 1 (of reference 1) contains more of the polar compounds like the azadirachtins and peak 2 also furnishes the same azadirachtins albeit in smaller amounts. The reason for the cross over of these compounds to peak 2 (reference 1) could be the high rate of elultion (30 mL/min) and the greater eluting capacity of the solvent employed (60:40 MeOH : H_2O). Peak 2 also contains more of the less polar compounds, whose identities have yet to be established.

REFERENCES

1. T. R. Govindachari, G. Suresh, G. Gopalakrishnan, J. Liq. Chrom., **18**, 3465-3471 (1995).

2. J. D. Stark, J. F. Walter, J. Agric. Biol. Chem., **43**, 507-512 (1995).

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Accepted November 6, 1995
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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIAZINON IN POLYMERIC MATRIX

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ABSTRACT

As a part of a study of pesticide migration through a polymer matrix, an HPLC method for analysis of O,O-diethyl-O-(6-methyl-2-(1-methylethyl)-4-pyrimidinyl)-phosphorothioate, commonly called diazinon) was developed.

INTRODUCTION

In human or animal therapy, some medicinal forms use a principle where the active compound is incorporated into a polymer matrix and gradually diffused following controllable kinetics. Control of the active ingredient at

different times during treatment is of great importance.[1-3] Also, realisation of this kind of presentation requires accurate and reliable analytical tools.

This paper describes an HPLC method for determination of an organophosphorus substance, O,O-diethyl-O-(6-methyl-2-(1-methylethyl)-4-pyrimidinyl)-phosphorothioate, commonly called diazinon. This compound is dispersed in a polyvinyl chloride polymer matrix. This kind of formulation, used in the pesticide field, should be considered as an interesting model for long-term treatment by the slow migration of an active substance.

Also, it is necessary, after extraction, to elaborate a chromatographic technique which allows a qualitative and quantitative evaluation. This was achieved by an internal standardization method.[4-8]

Currently, the most common analysis methods for determination of this compound use gas phase chromatography.[9-13] Gas chromatography presents some limitations in the case of a thermolabile substance (the degradation of diazinon begins at 120°C) and liquid chromatography appears to be more suitable. Most of the previous HPLC work on diazinon has been devoted to the environment, especially in water and vegetables.[14-16]

EXPERIMENTAL

Materials and Equipment

The HPLC system consisted of a Millipore Waters unit with a UV/Visible diode array detector (Waters 991), with detection at 250 nm. The analysis was carried out with an ODS C_{18} (250 mm x 4.6 mm I. D., 5 microns) reversed phase column. The mobile phase was a mixture of acetonitrile/water, 85/15 (v/v). The flow rate was 1 mL/min.

Preparation of Solutions

Internal standard solution.

Accurately weigh about 0.12 g of benzophenone into a 20 mL volumetric flask and dilute with dioxane.

Reagent: Benzophénone , purity > 99%, Fluka.

Diazinon standard solution.

Accurately weigh about 0.12 g of diazinon into a 25 mL volumetric flask and dilute with ethanol.

Reagent: Diazinon standard, purity > 99.1% ,Cluzeau, 33 Ste. Foy la Grande.

Calibration solutions.

Successively, accurately, measure 4.5 mL; 3.5 mL; 2.0 mL; 1.0 mL; 0.5 ml of the diazinon standard solution and introduce into separate 10 mL volumetric flasks. Next, add 0.4 mL of the internal standard solution and dilute to volume with a mixture of ethanol/THF 4/1 (v/v).

Method of Sample Preparation.

Polyvinyl chloride is used as the polymer matrix. PVC and stabilizer are mixed, at 80°C. Diazinon and plasticizer (dibutyl phtalate) are incorporated. The resulting mixture is then heated to 110°C. After cooling, this dry blend is extruded into a continuous strap into a cold water bath and dried in an air current.

Sample solution.

To 1 g of sample, add 10 mL of tetrahydrofuran. After total dissolution, add 35 ml of ethanol; the polyvinyl chloride precipitates. This preparation is filtered into a 100 mL volumetric flask. This operation is repeated a second time. Then add 4 mL of internal standard solution and dilute with ethanol to 100 mL.

Analysis was performed after preparation of a polymeric sample. This sample contained approximately 15% of diazinon. It corresponded to the veterinary licence quantity. This diazinon quantity should be contained between the limit values 13.5% and 16.5% in mass percentage.

The diazinon quantity incorporated was equal to 16%, an amount of active compound practiced to compensate for eventual loss of diazinon during the extrusion phase.

Determination of Diazinon Content.

P1 = diazinon standard purity,
Ws = diazinon weight in standard solution,
Wt = sample weight in sample solution,
We = benzophenone weight in diazinon standard solution,
W'e = benzophenone weight in sample solution,
Xs = benzophenone peak area obtained with diazinon standard solution,
Xt = benzophenone peak area obtained with a sample solution,
Ys = diazinon peak area obtained with diazinon standard solution,
Yt = diazinon peak area obtained with sample solution,
Rs = Ys/Xs ratio,
Rt = Yt/Xt ratio

The content of diazinon in the sample was calculated from the following equation:

$$\% \text{ Diazinon} = Rt/Rs \times ((Ws/We \times P1) / (Wt/W'e)) \times 100$$

RESULTS AND DISCUSSION

Presently, diazinon is analyzed by a gas chromatographic method. Diazinon is a thermolabile substance, its degradation temperature is 120°C. Hence, the gas chromatographic method is likely a destructive method.

The temperature conditions during GC analysis were 190°C for the column, 250°C for the injector and 280°C for the detector (FID). With this method, a good separation was obtained. The retention time for the benzophenone used as an internal standard was 2.63 minutes, for the diazinon, it was 3.30 minutes. A quantitative result was obtained; however, the diazinon was determined with a 4.3% variation coefficient (CV%). CV% was evaluated as an average after 10 injections. We obtained a low recovery.

In the literature, the referee CG method[10] gave the retention time for internal standard (Aldrin) at 10 to 12 minutes, for the diazinon, it was 5 to 6 minutes. Repeatability was equal to 1.9% and reproductibility to 2.0%.

The determination of diazinon by high performance liquid chromatography during preliminary analysis gives the retention time of benzophenone (t_R = 4.23 min) and Diazinon (t_R = 5.77 min). Using a diode array detector permits a tri-dimensional analysis in wavelength spectrum from

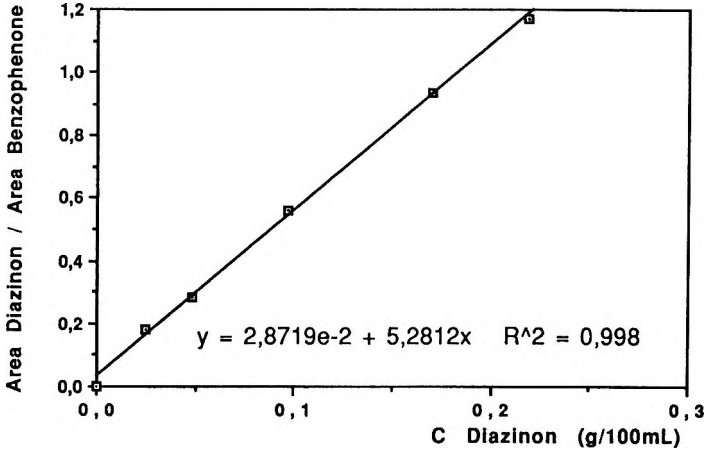

Figure 1. Calibration curve of diazinon. Graph representing peak area ratio of diazinon to benzophenone versus diazinon concentration.

180 nm to 380 nm. The wavelength selected was 250 nm in accordance with the absorption spectra of benzophenone and diazinon.

A calibration curve was established with a concentration range from 0.02 g/100 mL to 0.2 g/100 mL (three injections, 5 microliters each). For each concentration, the peak area ratio of diazinon to benzophenone Rs = $\Sigma n(Ys/Xs)/n$ were calculated. The results are reported in Table 1.

We have taken the detector response at a wavelength of 250 nm, for a diazinon range quantity from 0.2 µg to 200 µg. We have obtained a good linearity with a correlation coefficient of 0.998.

The precision of the analytical method was evaluated from the degree of repeatability of the peak area ratio of diazinon to an internal standard in a series of 10 injections. The precision of this method is usually expressed as the coefficient of variation (CV%).

Data reported in Table 2 are relative to a sample solution obtained after diazinon extraction from the polymeric matrix, as indicated in the experimental part.

Table 1

Calculation of Peak Area Ratio of Diazinon to Benzophenone

Std. Solution	Concentration (g/100mL)	Rs*
1	0.2187	1.167
2	0.1701	0.936
3	0.0972	0.560
4	0.0486	0.283
5	0.0243	0.178

* $Rs = \Sigma n(Ys/Xs)/n$

Table 2

Repeatability of Retention Times and Peak Area Ratios of Diazinon to Benzophenone*

	Benzophéone		Diazinon		R
Inj. No.	tR (min)	Xt	tR (min)	Yt	Yt/Xt
1	4.26	0.11831	5.84	0.07065	0.597
2	4.26	0.11899	5.83	0.07008	0.588
3	4.24	0.12353	5.81	0.07257	0.587
4	4.25	0.14002	5.79	0.08265	0.590
5	4.24	0.11543	5.78	0.06875	0.595
6	4.24	0.13314	5.79	0.07862	0.590
7	4.23	0.13197	5.75	0.07794	0.590
8	4.21	0.12485	5.73	0.07332	0.587
9	4.22	0.1283	5.74	0.07535	0.587
10	4.22	0.13364	5.74	0.0786	0.588
Average	4.237	0.12682	5.78	0.07485	0.590
CV %	0.46%	5.93%	0.69%	5.73%	0.56%

* Calculated from 10 analyses of a sample prepared as indicated in the Experimental section.

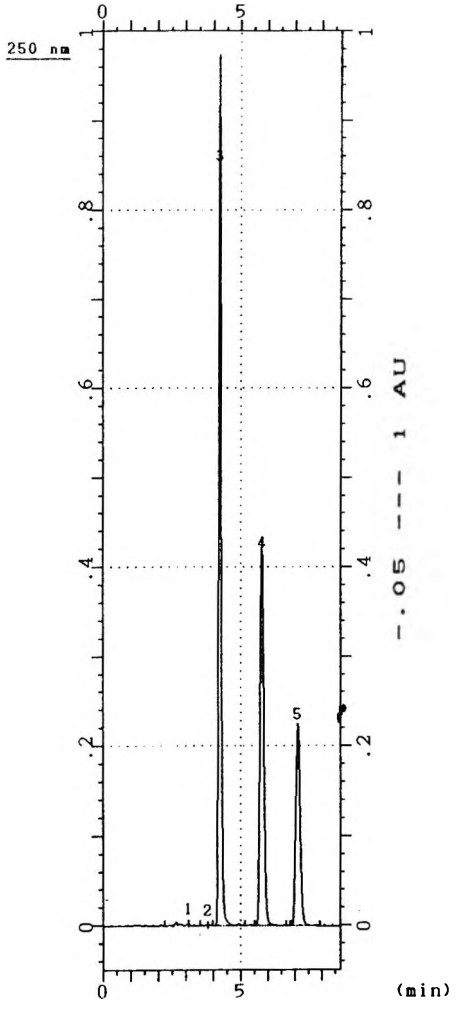

Figure 2. Chromatogram of benzophenone (3), diazinon (4) and plasticizer: dibutyl phtalate (5).

A good repeatability of retention times was obtained, $t_R = 4.23 \pm 0.02$ min for benzophenone and $t_R = 5.77 \pm 0.04$ min for diazinon. The coefficients of variation for the retention times are: 0.46% for benzophenone and 0.7% for diazinon.

The addition of an internal standard allowed us to neglect any error in the injected volume. After 10 injections, the precision of this method was evaluated from a ratio Yt/Xt of the variation coefficient and estimated at 0.56%. The active substance quantity in polymeric matrix has a mean value equal to 15.85 ± 0.08% w/w. It corresponds to a concentration range advocated by the veterinary license.

Figure 2 shows the chromatogram of a polymer sample analysis. Analysis conditions:

Column:	Kromasil® 110 ODS C_{18}
	250 mm x 4.6 mm I. D.
Mobil phase:	CH_3CN/H_2O, 85/15 v/v
Flow rate:	1 mL/mn
Detector:	UV/Visible diode array detector
Wavelength:	λ = 250 nm

CONCLUSION

This study shows the possibility of validating this HPLC method for qualitative and quantitative analysis of O,O-diethyl-O-(6-methyl-2-(1-methyl ethyl)-4- pyrimidinyl)-phosphorothioate (diazinon). The precision is evaluated by the coefficient of variation at 0.56%. The HPLC procedure developed appears to offer an attractive alternative approach to the currently employed gas chromatographic method in the veterinary license and CIPAC (Collaborative International Pesticides Analytical Council) referee method. This method presents a greater recovery and has the advantage of being very rapid. It is a non-destructive method and permits easy control of diazinon distribution kinetics during external therapeutic treatment.

REFERENCES

1. J. P. Varion, L. Yean, J. C Meslard, F. Subira, C. Bunel, L'Actualité Chimique, Sept./Oct., 330-335 (1992).

2. L. Cassiani-Ingoni, F. Subira, C. Bunel, J. P. Vairon, J. L. Halary, Makromol. Chem., Macromol. Symp., **19**, 287-293 (1988).

3. J. Grove, C. Bunuel, J. C. Meslard, J. P. Vairon, B. Plazonnet, **IVth World Conf. on Clin. Pharm. Therap.**, Mannheim (1989).

4. Peter L. Carter, Keith C. Overton, J. Assoc. Off. Anal. Chem., **69**, (5) 908-911 (1986).

5. U. Nemmer, N. Dimov, J. Chromatogr., **288**, 227-229 (1984).

6. Rice, L. Grant, J. Chromatogr., **317**, 523-526 (1984).

7. M. W. Dong, F. L. Vandermark, P. V. Passalacqua, LC Views, May, 1992.

8. R. Rosset, M. Caude, A. Jardy, **Manuel Pratique de Chromatographie en Phase Liquide**, 2nd edition, Masson, Paris, 1982.

9. Sétric International, Dossier Analytique AMM No. 69794100485.

10. CIPAC Method, **CIPAC Handbook**, Vol IA, p. 1199, 1980.

11. R. Turle, B. Levac, Bull. Environ. Contam. Toxicol., **38(5)**, 793 (1987).

12. A. F. Machin, C. R. Morris, Analyst, **97(1153)**, 289-293 (1972).

13. Karr J. James, J. Assoc. Off. Anal. Chem., **65(1)**, 115-119 (1982).

14. S. Farhat, M. M. H. Baig, M. A. Khan, Karachi Univ. J. Sci., **20(1-2)**, 143-149 (1992).

15. M. R. Driss, M. C. Hennion, M. L. Bouguerra, J. Chromatogr., **639(2)**, 352-358 (1993).

16. Mallet N. Victorin, M. Dugay, M. Bernier, N. Trottier, Int. J. Environ. Anal. Chem., **39(3)**, 271-279 (1990).

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MELATONIN DETERMINATION IN HUMAN URINE BY LIQUID CHROMATOGRAPHY USING FLUROMETRIC DETECTION

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ABSTRACT

A new, simple, very sensitive, highly reproducible, isocratic, and reverse phase liquid chromatographic method has been developed for determining urine melatonin concentrations using a fluorometric detector. 6-methoxymelatonin is used as an internal standard. A C_{18} column (150 X 0.46 cm) and a "Waters" fluorometric detector (Model 474 with a 16 uL flow cell are the main components of the liquid chromatographic system. The excitation and emission wavelengths are 285 and 335 nm respectively. Melatonin is extracted from urine at pH 7 using chloroform. The method is sensitive to 0.5 pg. The mean (n=6) inter-assay precision (CV) is 4.3% and intra-assay precision is 5.8%. The mean absolute recovery of the method is about 90%.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone of pineal origin. The circadian function of the pineal gland is reflected by the diurnal variation in the levels of melatonin in the blood. The highest level is noted in the night and the abnormal levels of the hormone are implicated in reproductive malfunctions and neurological and psychiatric disorders.[1] The extensive literature regarding the physiological and endocrinological role of melatonin[2] reveals the high level interest in the hormone.

There are many published methods for the analysis of melatonin in biological samples, including the radioimmunoassay (RIA),[3] gas chromatography-mass spectrometry,[4] and liquid chromatography.[5-9] The high performance liquid and chromatographic (HPLC) methods using fluorometric and electrochemical detectors are simple, cost effective, specific and seem to be more commonly used than the less sensitive RIA method. Most of the published HPLC methods do not use an internal standard, except one HPLC-UV method.[10] The utilization of an internal standard in an HPLC method involving multiple extraction and wash steps provides distinct advantages and enhances its quality. The use of 6-methoxy melatonin as an internal standard in the assay is a very good choice, because of its close structural similarity to melatonin, and no one has used the compound in the assay.

Careful study of the different HPLC analytical methods for melatonin reveals that the present methods totally lack the comprehensive features necessary for sensitive work. Our, new and simple, assay has been used to test clinically the validity of a hypothesis based on animal experiments, that idiopathic scoliosis may be reflected by the pineal gland function in adolescence.[11,12]

A total of 80 day time and night time, spot urine samples from both controls and patients were analyzed by the new HPLC method.

MATERIALS AND METHODS

Melatonin and other compounds used in interference studies in the method were obtained from Sigma, St. Louis, MO, USA; 6-methoxy melatonin was from Research Biochemicals, Inc., Natick, MA, USA. Monobasic potassium phosphate and triethylamine, and reagent grade chloroform, HPLC grade solvents acetonitrile and methanol were from Mallinkrodt Chemicals Co., Paris, KY, USA. All extractions were done in 25 mL all Teflon screw capped tubes (Nalgene) from VWR Scientific, Chicago, IL.

The HPLC system consisted of a pump by Hitachi (Danbury, CT, USA, Model L-6000), a C_{18}, 3 μm Econosphere column (150X 4.6 mm, Alltech Associates, Dearfield, IL, USA), an autosampler, from Waters (Model 717, Milford, MA, USA), a self packed guard column from Upchurch (Oak Harbor, WA, Model C-130-B) containing Perisorb RP-18 material, a fluorometric detector from Waters (Model 474) with a 16 μL flow cell and an integrator from Hitachi (Model D 2500). The excitation and emission wavelengths for the detector in the assay were 285 and 345 mm respectively.

The mobile phase is a mixture of phosphate (KH_2PO_4) buffer (30 mmol, containing 5 mL of triethylamine per litre and its pH adjusted to 4.3) acetonitrile and methanol (85 : 11 : 4 v/v). The flow rate is 1.35 mL/min.

Primary stock standard solutions of melatonin and 6-methoxy melatonin contained 1 mg of compound per mL of methanol solution. Secondary standards were prepared in 0.1% ascorbic acid solution. Aqueous working standards of melatonin containing 25, 62.5, 125, 250 pg/mL solution were prepared freshly ,on assay days, using a ng/mL standard. The internal standard solution (6-methoxymelatonin) contained 50 ng of compound per 50 μL of aqueous solution.

All primary and secondary standards were aliquotted and kept frozen at -80°C. These standards were found to be stable for 6 months.

Patient Samples

The study involved 9 female adolescent patients determined to have scoliosis and 18 healthy female controls of the same age group with no medical problems. All participants provided two spot urine samples, one of them was the evening void corresponding to day time melatonin production, and the second sample was the first morning void corresponding to over-night time melatonin production. All samples were preserved with 1g of boric acid and kept at -80°C until analysis.

Extraction

Pipet 8 mL of urine or aqueous standard or quality control sample into a 25 mL Teflon tube, adjust its pH to 7.0, and add 50 μL (50 ng) of 6-methoxymelatonin and 10 mL of chloroform. Cap the tubes and shake them in a horizontal shaker for 30 min. Centrifuge the tubes for 10 min and aspirate the top aqueous layer. Wash the chloroform extract successively with 8 mL

each of 0.1M sodium hydroxide and water. Agitate the tubes with wash reagent for 10 min, centrifuge and aspirate off the top aqueous layer. To the final chloroform solution add 250 μL of 0.05M methanolic hydrochloric acid, vortex and evaporate the chloroform under nitrogen in a 45°C water bath. Reconstitute the residue in 150 μL of mobile phase and inject 60 μL into the HPLC column.

METHOD DEVELOPMENT

Melatonin concentration in urine is of the order of a few pico grams per mL. A simple, very sensitive, and specific method is required. Both electrochemical[7,9] and fluorometric[5,8] detectors used in liquid chromatographic work, enables one to achieve the low detection limits. Urine is our choice specimen for this work, since, it provides a good mean concentration for melatonin produced both during day and over-night.

There are three advantages in choosing urine over plasma: one, it is easy to obtain, two, large volumes of sample (8-10 mL) can be used for extraction and, three, it reflects the mean concentration of melatonin produced over a period of several hours.[13]

Our attempts to use the electrochemical detector (Coulochem model 5100A by Environmental Science Associates, Bedford, MA, USA) in the work, proved very difficult because of the highly complex nature of the urine matrix and the high voltage setting required for the method. On the other hand, chromatograms of urine extracts obtained using fluorometric detectors are relatively less complex. We readily adjusted the mobile phase composition for our choice of C_{18} column, and fit the internal standard (6-methoxymelatonin) in the chromatogram in a location free of urine matrix interferences.

One of the most important requisites for achieving the low detection limits in this assay, is the use of a sensitive fluorometric detector. We found the Shimadzu fluorometric detector (Model RF 551 with a 12 μL flow cell) to be quite inadequate at the lower end of the detection limits of the assay. The "Waters" scanning fluorometric detector (Model 474 with a 16 μL flow cell), clearly enabled us the detection of less than 1 pg concentration of melatonin.

Direct injection of 0.5 pg of melatonin into the column gave a peak of 2 cm height at an attenuation of 1, and the noise to signal ratio was < 0.025. We found the "Waters" detector to be about forty times more sensitive than the Shimadzu RF 551 detector at its low sensitivity setting.

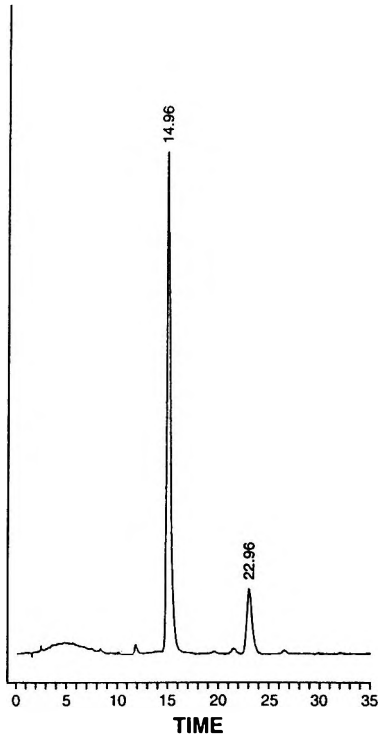

Figure 1. Chromatogram of an aqueous standard extract containing 125 pg/mL of melatonin and 50 ng of 6-methoxy melatonin.

Yet another important factor for the good sensitive nature of this assay method was our pH optimization studies of the extraction of melatonin and the internal standard from the aqueous standards and urine specimens. In this study, urine was adjusted to different pH values (4,6,7,8,12) and the analytes extracted using chloroform. At the neutral pH 7 the absolute extraction efficiency was found to be the greatest for both melatonin and 6-methoxymelatonin (mean 90%, n=6, refer Table 2 for relative recovery), and the chromatograms of urine extracts were cleanest. Although, at alkaline pHs the extraction efficiencies were equally good, the chromatograms were dirty and complex.

Our observations of good recovery from the urine at neutral and alkaline pHs is similar to earlier work on plasma samples.[6] Mills et al.[8] extracted melatonin from a saturated boric acid solution of urine and reported an absolute extraction efficiency of about 69%.

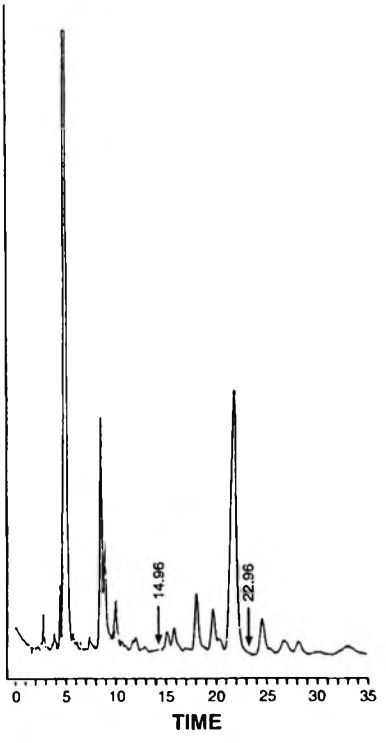

Figure 2. Chromatogram of an extract of urine stripped of melatonin and containing no 6-methoxymelatonin

RESULTS AND DISCUSSION

Representative chromatograms obtained in the work are shown in Figures 1-3. Figure 3 gives the chromatogram of a patient sample containing 57.6 pg/mL of melatonin, and Figure 2 is the chromatogram of stripped urine without the internal standard and shows the absence of interfering peaks at 14.9 min (retention time of 6-methoxymelatonin) and the blank spot at 22.96 min, the retention time of melatonin.

Table 1 gives a list of indole compounds that were checked for possible interferences in the work, and the study demonstrated the specificity of this method for melatonin. Mills et al.[8] in their work on urine samples, reported that 5-methoxy tryptophol and tryptophol eluted close to melatonin peak and our observations confirm the same. Table 2 gives details of the performance characteristics of the method. This precision and recovery

Table 1

Retention Times of Different Indole Compounds in the Assay

Compounds	Retention Times (min)
Serotonin	2.00
N-methylserotonin	2.10
5-Hydroxy tryptophan	2.10
L-Tryptophan	3.00
5-Methoxytryptophan	3.20
5-Hydroxy indole-3-aceticacid	3.76
5-Hydroxytryptophol	4.14
Indole-3-acetaldehyde	4.74
Tryptamine	4.89
5-Methoxytryptamine	5.00
N-acetylserotonin	5.19
N-methyltryptamine	5.64
6-Hydroxymelatonin	7.27
N-acetyl-L-tryptophan	7.77
6-Methoxymelatonin	14.91
5-Methoxy indole-3-acetic acid	15.42
Indole-3-acetic acid	15.47
5-Methoxytryptophol	17.70
Tryptophol	18.60
Melatonin	22.98

study was performed by initially stripping urine free of melatonin by repeated extractions (n=4) with chloroform, verifying its blank nature and then spiking it to known concentrations. Our mean (n=6) inter-assay coefficient of variation is 4.3%, and the mean intra-assay coefficient of variation is 5.8%. The mean (n=6) relative recovery (with respect to internal standard) of melatonin using the above spiked samples is 96%, and absolute recovery was 90% (n=6).

All sample measurements were made using a series of aqueous standards covering the range 25 to 250 pg/mL, and the method is linear in the concentration range 0 to 1000 pg. Standard curves were obtained using regression method, and the mean correlation coefficient for the line was 0.996. A detailed report of the sample results obtained by the method will be published elsewhere.[12] Table 3 gives the results for both the evening and morning samples of patients in a condensed form.

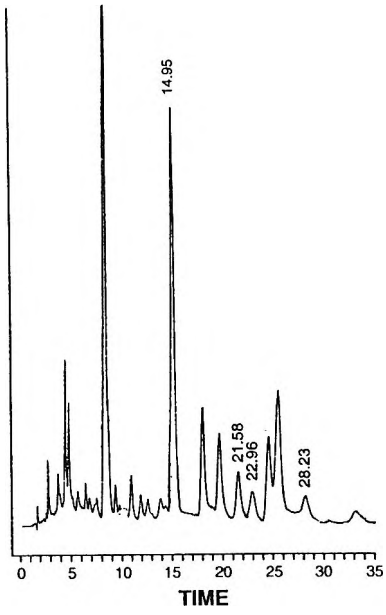

Figure 3. Chromatogram of a patient urine extract containing 57.6 pg/mL of melatonin and 50 ng of 6-methoxymelatonin

Table 4 gives a short summary and the detection limits of some of the recent HPLC electrochemical or fluorometric detector methods for the assay of melatonin. Two different claims[6,9] have been made using the same and efficient electrochemical detector (Environmental Science Associates, Bedford, MA). There is only limited scope to improve the detection limit using even an efficient electrochemical detector. The factors to achieve this include, using larger sample volumes for extraction, smaller size (internal diameter) columns, mobile phase composition, column heating, increase in mobile phase buffer concentration, and addition of ion-pairing reagents. But, with a fluorometric detector, the scope for improved detection limits are much greater because there are many other factors besides those listed above. In a dilute solution, the magnitude of fluorescence intensity depends upon the concentration of the fluorophor, the path length of the flow cell, the intensity of the exciting radiation, absortivity of the substance and its quantum efficiency.[13] Many workers, familiar with amino acids analyses, know that the sensitivity of fluorescent tag method reaches the femto moles range, whereas, with the electrochemical method it is in the range of pico moles. Another advantage with a fluorometric detector is that the baseline stabilizes very fast and it could

Table 2

Precision and Recovery Data for Melatonin Using Stripped Urine

Concn. Added	Conc. Measured Mean * ± S.D. (pg/mL)	C.V. %	Recovery Mean* %
	Inter-assay		
25	23 ± 1.15	5.0	92
62.5	60 ± 2.76	4.6	96
125	120 ± 4.32	3.6	96
250	237 ± 9.48	4.0	95
	Intra-assay		
25	22 ± 1.98	9.8	88
62.5	64 ± 3.07	4.8	102
125	128 ± 5.25	4.1	102
250	245 ± 11.0	4.5	98

* n = 6

Table 3

Urine Melatonin Concentrations in Our Patients

Mel. Concn. Range pg/mL	Frequency of Repetitions AM Results	PM Results
5 - 10	0	9
11 - 20	0	12
21 - 35	0	3
36 - 50	6	1
51 - 75	7	1
76 - 120	6	1
121 - 180	4	1
181 - 280	3	0

Table 4

Highlights of a Few Recent HPLC EC/FLU Methods for Melatonin Assay

Ref	Detector	DL	Samp	Recov %	Remarks
9	EC-ESA	1pg	2mL	90	Cell was set at (0.5 mV). Not the optimum potl.
6	EC-ESA	4pg	NK	97	Cell at optimum potl. 0.75 mV
7	EC-amp	15pg	2mL	78	Col 5 x 0.46, RT mel 25 min
8	FLU	30pg	8mL	69	Col 25 x 0.46, at 60°C, urine pH not optimized for extraction
5	FLU	6 pg	4mL	76	Gradient method; col at 30°C
*	FLU	0.5Pg	8mL	96	"Waters" Flu detector (Model 474, with a 16 μL flow cell) internal standard method

amp: amperometric detector; Col: column; DL: detection limit; NK: not known; potl.: potential; RT: retention time; *this method.

take several hours to stabilize an electrochemical detector. Thus the careful selection of a very good fluorometric detector for the melatonin assay is one of the main keys for a successful and sensitive HPLC method. We found that the fluorometric detector by "Waters" Model 474 with a 16 μL flow cell to be one of the best, and the on column detection limit for melatonin in our method is 0.5 pg (gave a peak height of 2 cm at an attenuation of 1).

The two recent fluorometric methods[5,8] for melatonin have other drawbacks besides the lack of sensitivity of their fluorometric detector. Both of them do not use an internal standard. Also, Penniston et al.'s method is a gradient method, and the run time per sample is 48 min, and the column is heated to 30°C, and the recovery of their solid phase extraction method is ~70%. Mills et al.[8] heated their column to 60°C.

In summary, when there is a choice between fluorometric and electrochemical detection for a compound, the former is far superior and several orders of magnitude more sensitive than the latter and easier to use. Our fluorometric HPLC assay for melatonin improves upon the earlier ones[5,8] by using a very sensitive detector and a good internal standard. The method has good accuracy (96%) and precision (C.V. ~5%).

REFERENCES

1. S. Binkley, **The Pineal: Endocrine and Nonendocrine Function**, Prentice-Hall, New Jersey, 1988.

2. A. Miles, D. Philbrick, CRC Crit. Rev. Clin. Lab. Sci., **25**, 231-253 (1987).

3. B. Manz, A. Seidel, H. Alexander, L. Vollrath, B. Wagner, G. Zimmerman, J. Clin. Chem. Clin. Biochem., **27**, 797-802 (1989).

4. S. F. Pang, C. W. Tsang, G. X. Hong, P. C. Yip, P. L. Tang, G. M. Brown, J. Pineal Res., **8**, 179-192 (1990).

5. J. F. Peniston-Bird, W. L. Di, C. A. Street, A. Kadva, M. A. Stalteri, R. E. Silman, Clin. Chem., **39**, 2242-2247 (1993).

6. F. Raynaud, P. Pevet, J. Chromatogr., **564**, 103-113 (1991).

7. Y. Sagara, Y. Okatani, S. Yamanaka, T. Kiriyama, J. Chromatogr., **431**, 170-176 (1988).

8. M. H. Mills, M. G. King, N. G. Keats, R. A. McDonald, J. Chromatogr., **377**, 350-355 (1986).

9. R. Vieira, J. Miguez, M. Lema, M. Aldegunde, Annal Biochem., **205**, 300-305 (1992).

10. K. Yamada, Y. Sakamoto, T. Satoh, Res. Commun. Chem. Path. and Pharm., **46**, 283-287 (1984).

11. M. Machida, J. Dubousset, Y. Imamira, T. Iwaya, T. Yamada, J. Kimura, Spine, **18**, 1609-1613 (1993).

12. A. S. Hilibrand, L. S. Blakemore, R. T. Loder, M. L. Greenfield, F. A. Farley, R. N. Hensinger, M. Hariharan, Spine, in press.

13. H. J. Lynch, R. J. Wurtman, M. S. Moskowitz, M. C. Archer, M. H. Ho, Science, **187**, 169-171 (1975).

14. **Instruction Manual (1993) Model F-1080 fluorescence Detector**, Hitachi Instruments, Inc., San Jose, CA, p. 5-2.

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INFLUENCE OF ALCOHOL ORGANIC MODIFIERS UPON THE ASSOCIATION CONSTANTS AND RETENTION MECHANISM FOR AROMATIC COMPOUNDS IN MICELLAR LIQUID CHROMATOGRAPHY

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ABSTRACT

Solute-micelle association constants and partition coefficients between stationary, aqueous, and micellar phases have been determined for a group of benzene derivatives and polycyclic aromatic hydrocarbons with sodium dodecyl sulphate and hexadecyltrimethylammonium bromide by Micellar Liquid Chromatography (MLC) with an octylsilica column using micellar mobile phases in absence and in the presence of organic modifiers (methanol, n-propanol, and n-butanol). The retention mechanism of these compounds in the chromatographic system has been studied by comparing experimental capacity factors and selectivity coefficients with those theoretically calculated assuming a direct transfer mechanism. When the surfactant concentration in the mobile phase is increased, a tendency to a change from a three-partition equilibrium mechanism to a direct transfer of solutes from micellar to stationary phase is observed for both surfactants. This change was favored when: (1) the hydrophobic character of the solute increased, (2) CTAB was used as surfactant, and (3) the

polarity of the aqueous mobile phase was increased. For highly hydrophobic compounds experiencing a direct transfer mechanism, selectivity coefficients for a pair of solutes can be predicted from the ratio of two stationary-micellarpartition coefficients.

INTRODUCTION

One application of Micellar Liquid Chromatography (MLC), has been the determination of solute-micelle association constants of great interest in chemistry and other disciplines.[1] This technique has been useful to determine these association constants for numerous organic compounds with different purely and modified micellar systems being salts and alcohols the most used modifiers.[2-12]

Solute-micelle association constants can be used to facilitate the systematic optimization in MLC.[10] In fact, the separation possibilities for a group of compounds can be forseen if their solute-micelle association constants are known. This aspect is very important, due to the unique selectivities obtained in MLC. Several articles have been published on the selectivity control in MLC through modification of the mobile phase composition. The effects, after addition of organic modifiers to micellar eluents, on solute retention, solvent strength, and selectivity have been studied.[13-18]

Gradient elution in MLC has been discussed recently.[19,20] Organic modifiers can improve separation selectivity but also increase efficiency, which is generally minor with micellar than with hydro-organic mobile phases, due to poor wetting of the stationary phase and restricted mass transfer.[21,22]

It was shown that the addition of a short- or medium-chain alcohol causes surfactant desorption from the stationary phase and improves efficiency.[23] Surfactant adsorption on the stationary phase not only has a great impact on the efficiency,[24,25] but also on the selectivity obtained in MLC. In fact, some studies have been recently made concerning the stationary phase effects on retention behavior and selectivity in MLC,[26,27] as well as on the effects of surfactant adsorption in MLC.[28]

In some works, solute-micelle association constants and partition coefficients have been used to better understand the differences in selectivity for various micellar systems,[27] or to study the retention mechanism in MLC and separation selectivity implications.[29]

Table 1

Experimental Conditions in which the Capacity Factors for the 23 Compounds have been Studied

Surfactant	Conc'n (M)	Alcohol	%	Solutes	C.M.C. (M) (ref. 34)
SDS	0.035; 0.050; 0.067 0.080; 0.100; 0.120	none	none	1-15	$8.00*10^{-3}$
SDS	0.035; 0.050; 0.067 0.080; 0.100; 0.120	methanol	10%	1-23	$8.00*10^{-3}$
SDS	0.050; 0.067; 0.080 0.100; 0.120; 0.140	n-propanol	3%	1-23	$8.00*10^{-3}$
SDS	0.050; 0.067; 0.080 0.100; 0.120; 0.140	n-propanol	5%	1-23	$7.00*10^{-3}$
SDS	0.035; 0.050; 0.067 0.080; 0.100; 0.120	n-propanol	10%	1-23	$3.50*10^{-3}$
SDS	0.050; 0.067; 0.080 0.100; 0.120; 0.140	n-butanol	3%	1-23	$5.00*10^{-3}$
SDS	0.050; 0.067; 0.080 0.100; 0.120; 0.140	n-butanol	5%	1-23	$3.50*10^{-3}$
SDS	0.050; 0.067; 0.080 0.100; 0.120; 0.140	n-butanol	10%	1-23	$1.25*10^{-2}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	none	none	1-15	$9.50*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-propanol	3%	1-23	$9.00*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-propanol	5%	1-23	$7.00*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-propanol	10%	1-23	$3.50*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-butanol	3%	1-23	$5.00*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-butanol	5%	1-23	$2.25*10^{-4}$
CTAB	0.050; 0.067; 0.080 0.100; 0.120	n-butanol	10%	1-23	$8.45*10^{-3}$

In this work, the retention mechanism and separation selectivity have been studied for a group of benzene derivatives and polycyclic aromatic hydrocarbons in an MLC system in which sodium dodecyl sulphate (SDS) and hexadecyltrimethylammoniumbromide (CTAB) were used as surfactants. Solute-micelle association constants and partition coefficients were previously determined and used to study the retention mechanism.

EXPERIMENTAL

Benzene derivatives and polycyclic aromatic hydrocarbons were (1) benzene, (2) benzyl alcohol, (3) benzamide, (4) toluene, (5) benzonitrile, (6) nitrobenzene, (7) phenol, (8) 2-phenylethanol, (9) chlorobenzene, (10) phenylacetonitrile, (11) 3,5-dimethylphenol, (12) naphthalene, (13) 1-naphthol, (14) 2-naphthol, (15) 1-naphthylamine, (16) pyrene, (17) phenanthrene, (18) 2,3-benzofluorene, (19) fluorene, (20) fluoranthene, (21) acenaphthylene, (22) acenaphthene, and (23) anthracene.

Experimental chromatographic data used in this work were previously determined in ref. 30 in the case of fifteen benzene and naphthalene derivatives and in ref. 31 in the case of polycyclic aromatic hydrocarbons.

Table 1 groups the experimental conditions in which the capacity factors for the 23 compounds were determined. Column void volumes of 1.09 mL for SDS and 0.97 mL for CTAB were used for all capacity factor calculations. Capacity factor values employed were averages of at least three determinations. Relative errors in determining the association constants were ascertained from the statistical parameters of the least-squares fitting and from error propagation.[32]

For the determination of solute-micelle association constants the following equation[2] was used:

$$Vs / (Ve-Vm) = v (Pmw-1) C_m / Psw + 1 / Psw \qquad (1)$$

where Vs, Ve, and Vm are the stationary phase volume, the solute elution volume, and the void volume of the columm respectively; Pmw is the solute partition coefficient between the micellar and aqueous phases, Psw is the partition coefficient of the solute between the stationary and aqueous phases, v is the surfactant molar volume, and C_m is the micellized surfactant concentration in the mobile phase (C_m = C - c.m.c., C being the total surfactant concentration in solution and c.m.c. the critical micelle concentration of the surfactant).

Table 2

Association Constants (and relative errors in %) Calculated from Eq. (1) for the 23 Compounds Studied and SDS Mobile Phases in Absence and in the Presence of Alcohols

No	SDS	SDS 10% MeOH	SDS 3% n-PrOH	SDS 5% n-PrOH	SDS 10% n-PrOH	SDS 3% n-BuOH	SDS 5% n-BuOH	SDS 10% n-BuOH
1	**23.52**	**17.43**	**19.10**	**15.86**	**16.88**	**16.49**	**13.28**	**10.94**
	(5.34)	(5.04)	(1.28)	(3.07)	(1.99)	(5.44)	(10.33)	(6.06)
2	**11.84**	**12.97**	**8.16**	**7.24**	**6.51**	**5.53**	**3.44**	**4.77**
	(5.06)	(8.17)	(4.01)	(18.99)	(4.59)	4.44)	(14.19)	(3.34)
3	**12.28**	**13.55**	**7.58**	**3.63**	**4.62**	**4.63**	**2.15**	**4.21**
	(6.37)	(7.39)	(5.74)	(6.08)	(7.77)	(7.20)	(49.62)	4.10)
4	**76.12**	**45.11**	**54.86**	**54.71**	**43.86**	**44.20**	**38.78**	**16.53**
	(14.00)	(4.94)	(3.86)	(12.99)	(8.94)	(10.61)	(4.93)	(7.82)
5	**20.39**	**20.21**	**14.16**	**11.07**	**10.59**	**8.99**	**6.41**	**7.23**
	(4.23)	(7.56)	(1.55)	(3.18)	(4.55)	(8.86)	(4.43)	(4.74)
6	**25.97**	**21.97**	**18.23**	**15.85**	**13.86**	**12.64**	**7.30**	**7.70**
	(6.29)	(3.18)	(2.59)	(8.57)	(4.07)	(6.64)	(6.66)	(6.66)
7	**10.47**	**12.14**	**9.41**	**7.61**	**8.83**	**6.60**	**5.94**	**6.43**
	(7.51)	(6.36)	(3.45)	(18.35)	(3.58)	(7.64)	(2.98)	(3.74)
8	**20.78**	**22.86**	**14.64**	**11.97**	**11.40**	**19.12**	**4.78**	**6.84**
	(3.17)	(8.71)	(2.31)	(12.50)	(4.42)	(9.22)	(2.30)	(1.80)
9	**105.36**	**59.97**	**72.48**	**62.05**	**55.51**	**50.60**	**28.41**	**17.54**
	(17.64)	(5.62)	(5.50)	(6.46)	(9.90)	(9.70)	(6.29)	(8.57)
10	**30.73**	**33.96**	**21.14**	**14.42**	**14.47**	**12.40**	**6.64**	**7.53**
	(4.99)	(6.71)	(3.56)	(9.54)	(4.69)	(10.18)	(2.33)	(4.38)
11	**57.91**	**66.86**	**40.21**	**24.65**	**32.05**	**24.73**	**14.40**	**12.93**
	(13.57)	(3.40)	(2.82)	(17.99)	(5.03)	(10.11)	(13.85)	(6.75)
12	**217.10**	**465.93**	**226.71**	**207.22**	**117.75**	**96.87**	**24.04**	**21.23**
	(79.77)	(38.99)	(12.47)	(23.54)	(13.73)	(16.27)	(9.23)	(9.62)
13	**189.54**	**123.98**	**79.79**	**51.98**	**62.09**	**44.74**	**25.28**	**17.60**
	(47.10)	(8.50)	(5.24)	(25.10)	(11.53)	(11.28)	(14.54)	(11.56)
14	**167.97**	**146.34**	**73.23**	**60.64**	**55.41**	**41.79**	**29.29**	**16.34**
	(40.11)	(20.01)	(5.40)	(15.07)	(10.67)	(11.74)	(13.98)	(9.37)
15	**248.00**	--	**62.19**	**44.57**	**35.26**	**31.95**	**18.38**	**12.90**
	(74.65)		(3.73)	(19.74)	(7.32)	(8.29)	(8.63)	(12.44)
16	--	--	--	**414.63**	--	**196.86**	**67.62**	**22.95**
				(97.98)		(47.97)	(13.72)	(13.97)
17	--	--	**63.33**	**161.60**	--	**173.98**	**75.23**	**22.15**
			(35.60)	(43.24)		(40.26)	(25.23)	(13.34)
18	--	--	--	--	--	**458.01**	**93.25**	**26.37**
						(106.54)	(17.70)	(14.210
19	--	--	**1326.44**	**529.75**	--	**179.27**	**61.76**	**22.34**
			(320.53)	(62.78)		(34.25)	(23.63)	(11.91)
20	--	--	--	**376.37**	--	**229.17**	**77.94**	**25.02**
				(122.40)		(41.68)	(17.75)	(13.02)
21	--	--	**496.08**	**195.90**	--	**129.28**	**56.36**	**22.06**
			(26.44)	(21.49)		(21.14)	(16.26)	(11.86)
22	--	--	**380.90**	**190.14**	--	**249.30**	**71.40**	**23.07**
			(100.28)	(42.55)		(46.33)	(13.70)	(11.39)
23	--	--	--	**330.99**	--	**229.37**	**91.76**	**26.01**
				(70.14)		(38.51)	(24.05)	(11.27)

A plot of Vs / (Ve-Vm) versus C_m should be linear and the solute-micelle binding constant per surfactant monomer, K_2 = v(Pmw -1) (33) can be calculated from the slope:intercept ratio of the straight line. Psw can be obtained from the intercept and, if the surfactant molar volume v is known, Pmw can also be calculated. As molar volumes for SDS and CTAB, values of 0.246 and 0.364 L mol^{-1} have been taken respectively.[1]

RESULTS AND DISCUSSION

Solute-Micelle Association Constants

Capacity factors for the twenty three solutes studied were determined for eighty three different mobile phases corresponding to the experimental conditions detailed at Table 1. Capacity factors for the polycyclic aromatic hydrocarbons could not be measured in absence of alcohols due to the high retention that these compounds experienced in the stationary phase in these conditions. However, when an alcohol was added to the micellar mobile phase, the capacity factors for the twenty three compounds studied were measured.

From the variation of the volume term in Eq. (1) as a function of the micellized surfactant concentration in the mobile phase (total surfactant concentration minus c.m.c. value given at Table 1) it was possible to determine the values of K_2, Pmw, and Psw from the parameters of the straight line obtained for this variation except for those cases where a negative intercept was obtained. Psm was calculated as the ratio between Psw and Pmw.

Tables 2 and 3 group the values of the association constants with SDS (Table 2) and CTAB (Table 3) for those solutes for which a positive intercept is obtained (Eq. (1)). These Tables give the values for the association constants and the relative errors obtained in absence of additives, in the presence of alcohols.

The variation of the volume term with C_m (Figures not shown) allows to obtain a straight line with a good correlation coefficient (in all cases it was higher than 0.99). Negative intercepts have already been obtained by other authors[9,35] and attributed to the error in the determination of K_2 for very hydrophobic compounds experiencing a high retention in the system and for which intercepts close to zero are obtained. Tables 4 and 5 group the values obtained for Psw for the solutes studied when SDS (Table 4) and CTAB (Table 5) mobile phases are used in absence of additives and in the presence of alcohols.

Table 3

Association Constants (and relative errors in %) Calculated from Eq. (1) for the 23 Compounds Studied and CTAB Mobile Phases in Absence and in the Presence of Alcohols

No	CTAB	CTAB 3% n-PrOH	CTAB 5% n-PrOH	CTAB 10% n-PrOH	CTAB 3% n-BuOH	CTAB 5% n-BuOH	CTAB 10% n-BuOH
1	**47.20**	**47.29**	**45.64**	**37.39**	**36.91**	**23.11**	**17.34**
	(2.10)	(2.07)	(7.20)	(3.23)	(4.36)	(4.37)	(1.350
2	**17.31**	**14.18**	**11.55**	**9.00**	**9.18**	**7.77**	**5.17**
	(3.08)	(4.60)	(3.55)	(2.82)	(7.92)	(1.44)	(8.40)
3	**12.43**	**10.05**	**7.66**	**6.95**	**8.23**	**5.53**	**3.04**
	(5.28)	(6.62)	(4.80)	(3.20)	(6.47)	(2.09)	(3.34)
4	**203.88**	**281.18**	**203.33**	**126.78**	**104.09**	**45.49**	**25.29**
	(9.41)	(30.89)	(18.20)	(8.75)	(15.46)	(5.39)	(4.07)
5	**27.17**	**24.70**	**19.19**	**16.85**	**20.72**	**8.83**	**9.00**
	(4.20)	(4.93)	(7.18)	(1.41)	(5.86)	(1.80)	(3.84)
6	**54.71**	**43.19**	**37.03**	**30.43**	**29.41**	**15.84**	**12.39**
	(3.48)	(2.90)	(12.47)	(2.34)	(3.18)	(3.15)	(2.97)
7	**79.53**	**60.48**	**37.68**	**26.78**	**22.88**	**8.91**	**11.63**
	(13.27)	(2.00)	(19.55)	(6.18)	(3.63)	(6.98)	(1.340
8	**30.02**	**23.36**	**19.18**	**15.09**	**16.75**	**9.01**	**7.97**
	(3.13)	(6.36)	(5.41)	(3.04)	(2.85)	(4.30)	(3.27)
9	**547.93**	**1434.12**	**431.94**	**234.09**	**132.94**	**54.45**	**28.16**
	(11.39)	(128.18)	(36.51)	(16.49)	(14.89)	(6.28)	(3.89)
10	**51.14**	**43.28**	**31.68**	**24.45**	**27.37**	**13.07**	**9.53**
	(5.48)	(3.30)	(12.70)	(3.42)	(7.86)	(5.43)	(3.60)
11	--	--	**225.94**	**161.01**	**69.39**	**24.06**	**25.77**
			(60.23)	(5.98)	(1.56)	(9.60)	(4.02)
12	--	--	--	--	**256.52**	**70.06**	**39.59**
					(23.83)	(13.46)	(6.71)
13	--	--	--	**779.81**	**120.05**	**33.20**	**62.06**
				(5.64)	(7.91)	(8.12)	915.54)
14	--	--	--	**597.85**	**104.54**	**30.62**	**45.33**
				(26.92)	(9.38)	(13.14)	(6.36)
15	--	--	**663.12**	**195.11**	**88.59**	**37.16**	**26.25**
			(89.31)	914.83)	(6.49)	(3.50)	(0.49)
16	--	--	--	--	**1173.87**	**98.35**	**67.40**
					(68.27)	(6.76)	(15.97)
17	--	--	--	--	**663.55**	**103.80**	**59.83**
					(39.46)	(4.10)	(13.98)
18	--	--	--	--	--	**100.24**	**69.62**
						(11.11)	(15.50)
19	--	--	--	--	**838.22**	**98.59**	**48.27**
					(82.53)	(13.58)	(9.88)
20	--	--	--	--	**970.01**	**98.39**	**71.42**
					(139.04)	(21.72)	(15.01)
21	--	--	--	--	**384.98**	**79.12**	**46.86**
					(37.91)	(16.88)	(8.87)
22	--	--	--	--	**828.42**	**59.20**	**43.31**
					(101.97)	(19.36)	(7.15)
23	--	--	--	--	**841.86**	**89.42**	**60.96**
					(63.88)	(18.13)	(11.21)

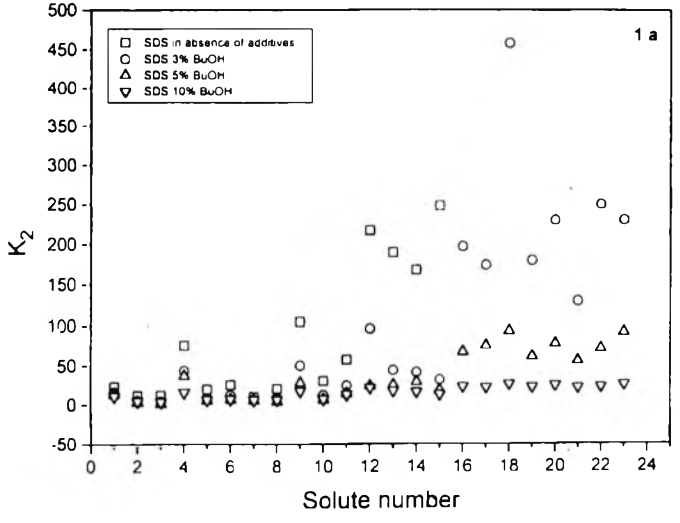
From Tables 2 and 3, the influence of the surfactant nature, and the nature and concentration of the alcohol added to the mobile phase on the association constants can be studied.

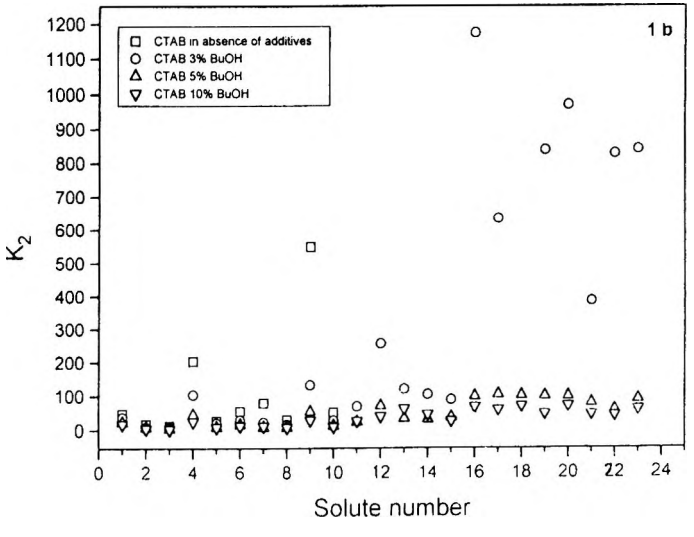
In regards to the surfactant nature, the association constants of the solutes are generally higher with CTAB than with SDS. This is due to the electrostatic interaction between the positively charged CTAB micelles and the unlocated charge of the solutes aromatic rings.

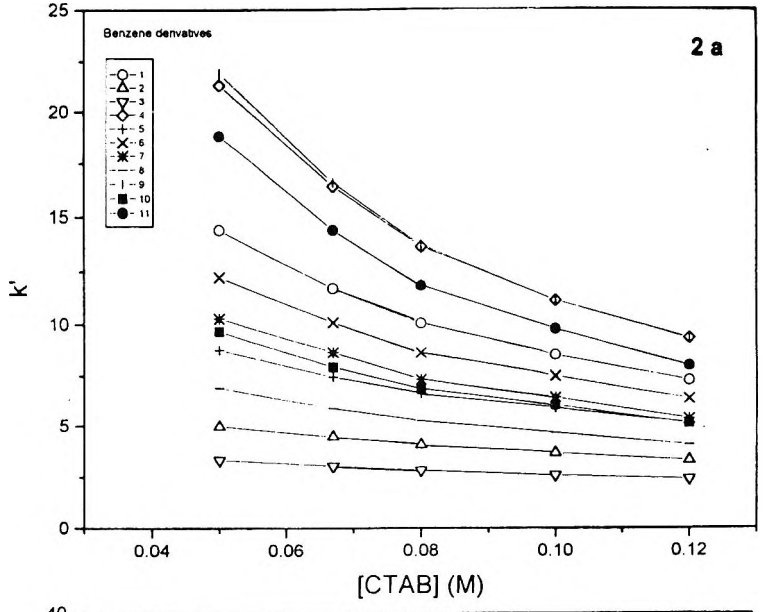
The addition of an alcohol generally decreases the solute-micelle association constant regardless of the surfactant nature. In fact, the presence of an alcohol in the mobile phase decreases the adsorption of the surfactant in the stationary phase[23] and the polarity of the aqueous nonmicellar phase, allowing to increase the affinity of the solute for the aqueous nonmicellar phase and decreasing its association with the micelle. This result is illustrated in Figure 1 which shows the association constants for the fifteen benzene and naphthalene derivatives with SDS (Figure 1a) and CTAB (Figure 1b) with and without butanol and for the polycyclic aromatic hydrocarbons in the presence of butanol.

For the compounds for which the comparison is possible, that is, for the fifteen benzene and naphthalene derivatives, it can be observed that the addition of butanol to the mobile phase decreases the solutes association constants with CTAB as well as with SDS. The same result is observed when adding n-propanol but in this case (as can be observed in Tables 2 and 3) there are more instances where the intercept of the straight line obtained for the variation of the retention term as a function of the micellized surfactant concentration is negative. The decrease in the association constant observed due to the addition of an alcohol to the mobile phase is higher for most hydrophobic compounds. Figures 1a and 1b also allow to study the influence of the alcohol percentage on the association constant values. These constants decrease when the alcohol percentage in the mobile phase increases. This also occurs with n-propanol. Such effect is also more obvious for compounds of a higher hydrophobic character. This makes it possible to ascertain solute-micelle association constants for some compounds for which such determination would be not possible in other conditions (in absence of alcohol or in the presence of small amounts of alcohol). The only instance in which this effect is not well appreciated is for SDS-n-propanol but this could be because the errors in the association constants for polycyclic aromatic hydrocarbons are the highest in this case.

Figure 1 (right). Solute-micelle association constants for the compounds studied with SDS and CTAB in absence of additives and in presence of 3%, 5%, and 10% n-butanol. a) SDS. b) CTAB.


SDS in absence of additives
SDS 3% BuOH
SDS 5% BuOH
SDS 10% BuOH
1 a
K2
Solute number


CTAB in absence of additives
CTAB 3% BuOH
CTAB 5% BuOH
CTAB 10% BuOH
1 b
K2
Solute number


Benzene derivatives
2 a
1
2
3
4
5
6
7
8
9
10
11
K'
25
20
15
10
5
0
0.04
0.06
0.08
0.10
0.12
[CTAB] (M)

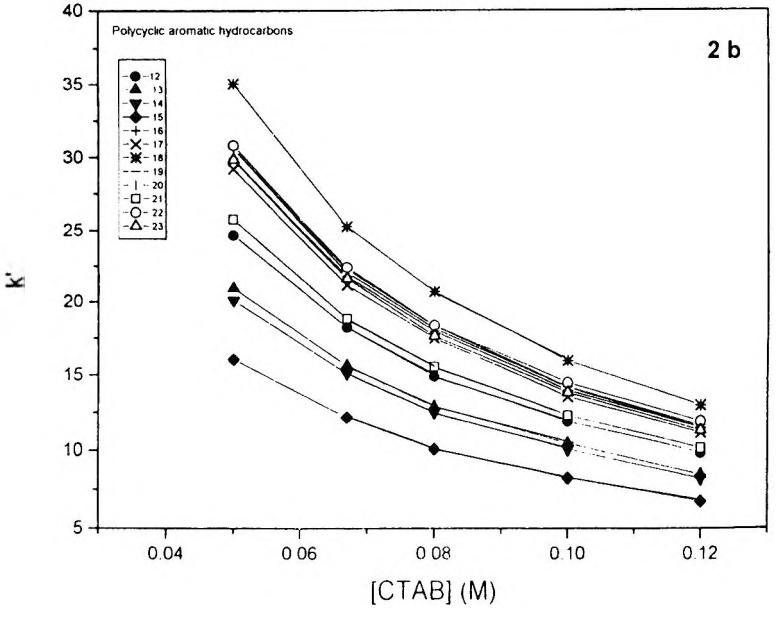

Polycyclic aromatic hydrocarbons
2 b
12
13
14
15
16
17
18
19
20
21
22
23
K'
40
35
30
25
20
15
10
5
0.04
0.06
0.08
0.10
0.12
[CTAB] (M)

Table 4

Solute Partition Coefficients Between the Stationary and Aqueous Phases, Psw, Calculated from Eq. (1) for SDS Mobile Phases in Absence and in the Presence of Alcohols

No	SDS	SDS 10% MeOH	SDS 3% n-PrOH	SDS 5% n-PrOH	SDS 10% n-PrOH	SDS 3% n-BuOH	SDS 5% n-BuOH	SDS 10% n-BuOH
1	51.64	31.65	34.49	34.15	26.10	26.78	24.90	12.45
2	12.09	7.98	7.06	5.55	3.99	4.42	3.56	2.32
3	9.96	6.55	5.31	3.52	2.45	3.09	2.32	1.46
4	198.45	95.35	112.44	124.01	75.52	78.32	71.09	20.03
5	30.56	21.09	18.40	14.60	10.51	11.31	89.46	6.07
6	46.55	29.55	27.42	24.38	16.77	17.48	13.81	8.18
7	12.03	8.29	7.97	7.05	5.35	5.39	5.29	3.39
8	24.86	17.02	14.19	11.07	7.63	8.11	5.86	3.66
9	289.03	127.60	149.98	144.05	93.03	91.42	60.03	21.38
10	42.22	31.23	24.70	17.93	12.26	13.03	9.29	5.36
11	90.23	68.98	50.55	33.06	28.50	27.55	18.94	9.74
12	715.48	1076.50	538.08	528.12	221.81	195.53	66.04	27.64
13	349.77	161.12	109.93	74.93	61.00	53.07	34.52	14.09
14	311.44	180.87	98.35	79.02	51.19	46.73	34.89	11.88
15	434.91	--	88.05	61.86	34.51	38.20	24.08	9.64
16	--	--	--	1606.20	--	552.14	197.03	34.97
17	--	--	281.56	591.54	--	445.23	202.99	32.71
18	--	--	--	--	--	1389.81	293.51	39.93
19	--	--	4086.15	1728.89	--	438.16	167.66	32.11
20	--	--	--	1453.78	--	637.41	225.59	36.93
21	--	--	1304.58	560.53	--	282.94	137.43	29.75
22	--	--	1118.70	637.84	--	582.80	183.26	32.65
23	--	--	--	1183.80	--	591.20	246.12	36.86

As could be expected, solute-micelle association constants decrease when the carbon atom number of the alcohol chain is increased due to the fact that an increase in the chain length causes a polarity decrease. This effect was observed for CTAB and SDS with n-propanol and n-butanol when comparing the three percentages studied and for SDS when comparing methanol, n-propanol, and n-butanol at 10 %.

Retention Mechanism and Separation Selectivity

Figure 2 shows the variation of the capacity factor as a function of the surfactant concentration for the benzene derivatives (Figure 2a) and for the polycyclic aromatic hydrocarbons (Figure 2b) with a CTAB - 5% n-propanol

Figure 2 (left). Variation of the experimental capacity factor of the compounds studied as a function of the total CTAB concentration in a mobile phase modified with 5% n-propanol. a) benzene derivatives. b) polycyclic aromatic hydrocarbons.

mobile phase. Similar results were obtained with all mobile phases, showing that separation selectivity generally increased when surfactant concentration was decreased in the mobile phase, this being true for both CTAB and SDS. Some authors[27] have explained this effect by stating that selectivity is primarily due to solute-stationary phase interactions, which are more favorable at lower surfactant concentrations. According to a previous paper,[36] SDS generally enhances separation selectivity with respect to CTAB and also the addition of a medium-chain alcohol, such as n-propanol or n-butanol, at medium percentages (3 or 5%) positively influences selectivity. Separation selectivity can be theoretically calculated for a group of compounds if a direct transfer mechanism of the solute between the stationary and micellar phases is assumed.[29] In fact, Borgerding et al.[35] have proposed a limit theory for those compounds whose affinity toward the micellar phase is large enough to experience a direct transfer from this phase to the stationary phase. These solutes only undergo a partition between the micellar pseudophase and the stationary phase since the solute amount in the aqueous phase is almost negligible. The capacity factor for these compounds can be expressed as:

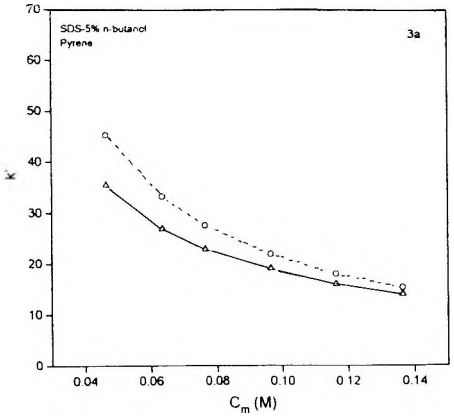
$$k' = Vs\ Psm / Vm\ v\ C_m \qquad (2)$$

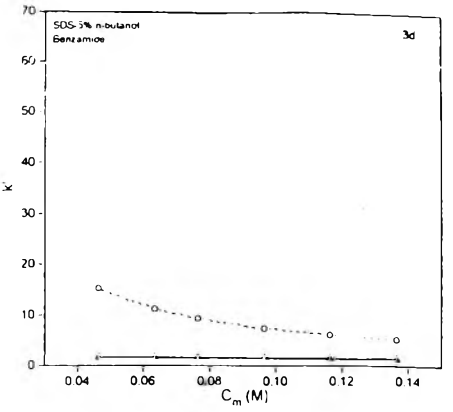
If this direct transfer mechanism is assumed for the solutes, and the surfactant concentration in the mobile phase is increased, the selectivity coefficient (I) for two compounds can be calculated from the distribution coefficients of the two solutes between the stationary and micellar phases:[29]

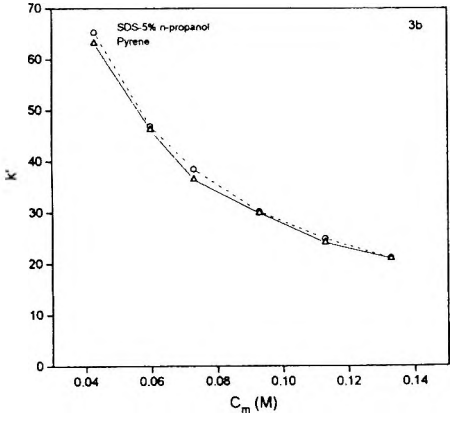
$$I = Psm_2 / Psm_1 \qquad (3)$$

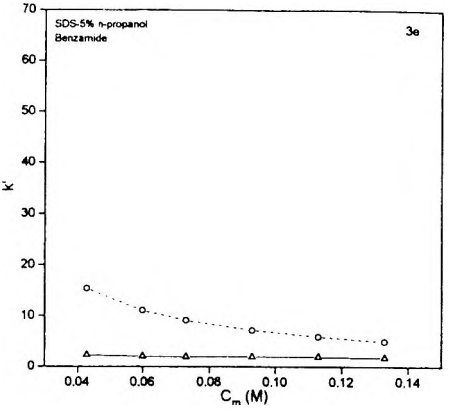
This equation shows that if this mechanism takes place, the separation selectivity will be constant and will not vary with the surfactant concentration in the mobile phase. Eqs. (2) and (3) have been used to calculate the capacity factor and selectivity coefficient for the compounds studied herein under different conditions. This has been made by using the partition coefficient, Psm, for each solute previously calculated (Psm = Psw / Pmw; Psw values are given in Tables 4 and 5; Pmw values can be calculated from K_2 values given in Tables 2 and 3 from the equation $K_2 = v\ (Pmw - 1)$ (33)). The agreement between the theoretical and experimental capacity factors and selectivity coefficients would indicate the experimental conditions in which a direct transfer mechanism for our compounds occurs.

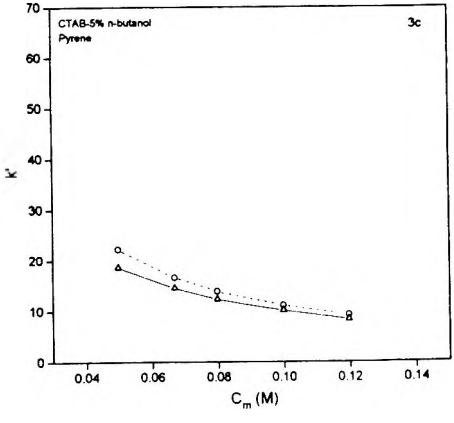
Figure 3 (right). Variation of experimental and theoretical capacity factors for pyrene and benzamide as a function of the micellized surfactant concentration in mobile phase. a) pyrene in SDS-5% n-butanol. b) pyrene in SDS-5% n-propanol. c) pyrene in CTAB-5% n-butanol. d) benzamide in SDS-5% n-butanol. e) benzamide in SDS-5% n-propanol. f) benzamide in CTAB-5% n-butanol.

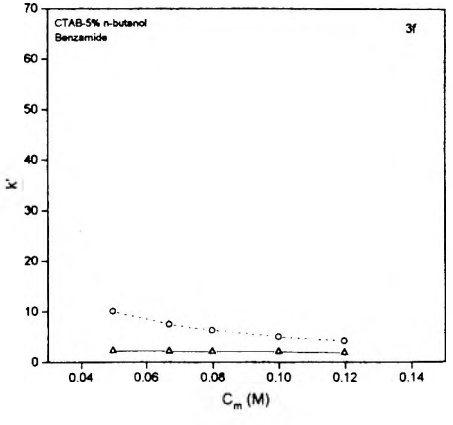




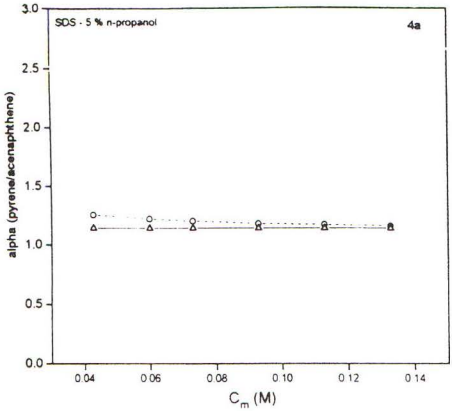


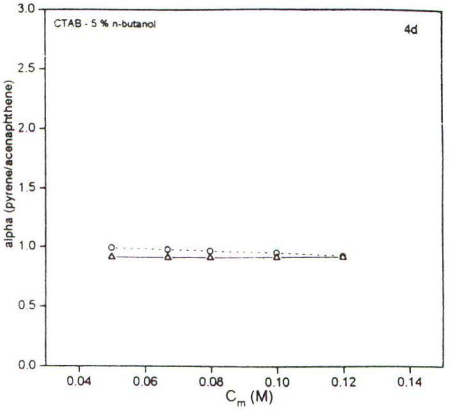


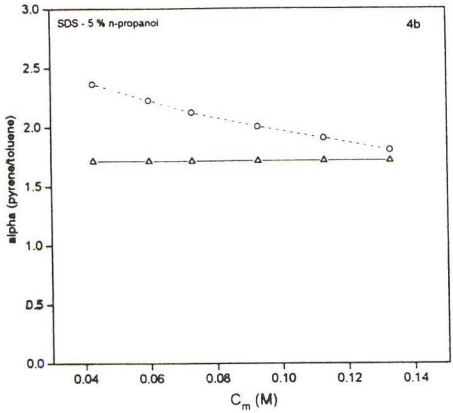


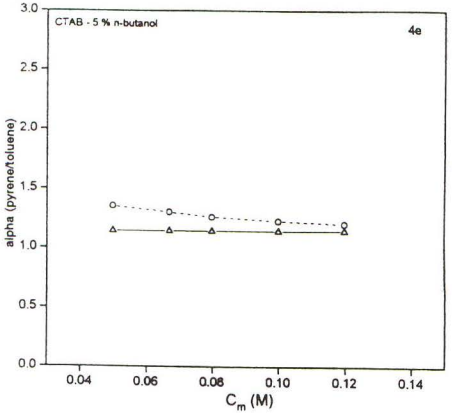


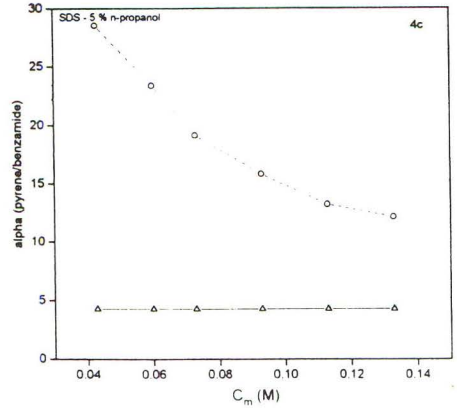
theoretical experimental

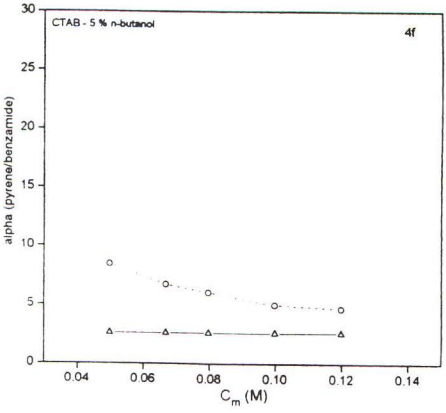












○ experimental alpha △ theoretical alpha

Figure 3 shows the variation of the theoretical and experimental capacity factor as a function of the micellized surfactant concentration for a very hydrophobic compound (pyrene) with a logarithm of the octanol-water partition coefficient, log P_{ow} = 4.88 and, for the lesser hydrophobic compound (benzamide) with log P_{ow} = 0.64. For each compound, the capacity factors are given for three mobile phases: SDS-5% n-butanol, SDS-5% n-propanol, and CTAB 5% n-butanol. This figure shows that the theoretical capacity factor is generally higher than the experimental one because the theoretical capacity factor is calculated from the partition coefficient between the stationary and the micellar phases, therefore neglecting the amount of solute in the aqueous nonmicellar phase. However, this partition between the aqueous and micellar phases is reflected in the experimental capacity factor. The difference between the theoretical and experimental capacity factors decreases when the surfactant concentration in solution for both solutes increases, allowing to obtain practically the same value for the capacity factor.

Consequently, it is possible to think that the three-partition equilibria mechanism changes to a direct transfer mechanism when the surfactant concentration is increased. The difference between the theoretical and experimental capacity factors does not depend only on the surfactant concentration, but also on the nature of the solute and the mobile phase. In fact, for the same mobile phase, this difference is less significant for pyrene than for benzamide, showing a lesser affinity of a highly hydrophobic compound for the aqueous nonmicellar phase and its higher affinity for the stationary phase. In regards to the nature of the mobile phase, when the polarity of the mobile phase is decreased by adding an alcohol, the affinity of a hydrophobic solute for the aqueous nonmicellar phase increases, subsequently increasing the difference between the theoretical and experimental capacity factors. This occurs in the case of pyrene when adding butanol to the mobile phase. The difference between the theoretical and experimental capacity factors for pyrene is minimum when using n-propanol in the mobile phase which has a higher polarity than n-butanol and also when using CTAB as surfactant. For a low hydrophobic compound such as benzamide, the nature of the alcohol does not influence, appreciably, the difference between the capacity factors, since it has a certain affinity for the aqueous nonmicellar mobile phase. However, this difference is minor for CTAB than for SDS since the higher association constant with CTAB decreases its affinity for the aqueous nonmicellar mobile phase.

Figure 4 (left). Variation of the experimental and theoretical selectivity coefficients (I) as a function of the micellized surfactant concentration for three pairs of solutes: pyrene-acenaphthene, pyrene-toluene and pyrene-benzamide. a), b), and c) SDS-5% n-propanol. d), e), and f) CTAB-5% n-butanol.

Table 5

Solute Partition Coefficients between the Stationary and Aqueous Phases, Psw, Calculated from Eq. (1) for CTAB Mobile Phases in Absence and in the Presence of Alcohols

No	CTAB	CTAB 3% n-PrOH	CTAB 5% n-PrOH	CTAB 10% n-PrOH	CTAB 3% n-BuOH	CTAB 5% n-BuOH	CTAB 10% n-BuOH
1	58.35	52.56	49.92	36.50	33.00	22.13	12.35
2	16.05	10.72	8.33	5.28	6.25	4.71	2.65
3	10.02	6.58	4.86	3.20	4.56	3.14	1.68
4	278.14	343.18	251.10	137.36	101.23	47.52	19.46
5	29.87	23.00	18.03	13.25	14.44	8.22	5.71
6	63.29	43.66	36.84	25.49	23.56	14.28	8.35
7	88.56	52.63	31.68	16.25	17.68	8.42	5.12
8	28.34	18.29	14.24	9.05	10.65	6.46	3.94
9	735.70	1720.23	520.33	243.06	129.67	55.75	21.30
10	51.71	36.35	26.17	16.42	18.09	9.80	5.40
11	--	--	242.97	124.14	61.94	23.15	12.84
12	--	--	--	--	257.80	74.46	29.01
13	--	--	--	597.84	112.14	32.04	27.54
14	--	--	--	443.43	97.70	29.69	20.57
15	--	--	580.49	130.06	69.37	28.74	12.80
16	--	--	--	--	1376.69	116.35	50.46
17	--	--	--	--	711.02	117.06	44.07
18	--	--	--	--	--	125.58	52.73
19	--	--	--	--	942.27	114.14	37.16
20	--	--	--	--	1121.99	114.75	52.02
21	--	--	--	--	395.56	85.19	33.96
22	--	--	--	--	944.61	77.18	34.87
23	--	--	--	--	944.86	103.69	44.91

From the results obtained on the capacity factors, it can be stated that a direct transfer mechanism occurs for solutes in MLC when increasing the hydrophobicity of the solute, the surfactant concentration, and when using CTAB instead of SDS and the polarity of the alcohol in the mobile phase increases. These results would explain the fact that most of the negative intercepts or the highest errors in the determination of the association constants for the highest hydrophobic compounds are obtained when CTAB is the surfactant or n-propanol is the organic modifier in mobile phase (see Tables 2 and 3).

In fact, when a direct transfer mechanism occurs, an intercept close to zero should be obtained (Eq. 2) for the variation of the inverse of the capacity factor as a function of C_m. Consequently, the calculation of the association constant in these conditions would not be adequate due to the high error associated to this calculation.

In those cases where the theoretical and experimental capacity factors are similar for two solutes, that is, when a direct transfer mechanism occurs, the selectivity coefficient can be forseen from the ratio between the solutes partition coefficients between the stationary and micellar phases (Eq. 3). Figure 4 shows the variation of the theoretical and experimental selectivity coefficient as a function of the surfactant concentration in a SDS-5% n-propanol (Figures 4a, 4b, and 4c) and in a CTAB-5% n-butanol mobile phase (Figures 4d, 4e, and 4f) for three pairs of solutes: pyrene- acenaphthene which are both very hydrophobic and for which a direct transfer mechanism can be assumed for any surfactant concentration in these mobile phases, pyrene-toluene in which only for pyrene a direct transfer mechanism can be assumed for all surfactant concentrations, and pyrene-benzamide in which benzamide does not experience a direct transfer mechanism except at very high surfactant concentrations. Figure 4 shows that, when both solutes experience a direct transfer mechanism, the experimental and theoretical selectivity coefficients are very similar for all surfactant concentrations in solution, being therefore possible to predict the selectivity coefficient from the partition coefficients Psm for the two solutes. When one of the two solutes does not experience a direct transfer mechanism, the theoretical and experimental selectivity are different and this difference decreases under the same conditions in which the direct transfer mechanism is favored, that is, by increasing the hydrophobicity of the solute, the surfactant concentration, the polarity of the alcohol in mobile phase, and also using CTAB as surfactant.

CONCLUSIONS

From the results obtained in this work for the twenty three benzene derivatives and polycyclic aromatic hydrocarbons studied, it can be concluded that, when the surfactant concentration in mobile phase is increased, there is a progressive tendency to change from a three-partition equilibria mechanism to a direct transfer of solutes from micelles to the stationary phase. This tendency is higher when increasing solute-micelle association constants, that is, when the hydrophobicity of the solute is increased, CTAB is used as surfactant, and the length of the alcohol chain in the mobile phase decreases. Consequently, the separation selectivity for a pair of solutes also shows a tendency to match a limit value when the surfactant concentration in solution is increased. This limit value is close to the ratio of stationary-micellar partition coefficients of two solutes and does not depend on the surfactant concentration in solution.

For very hydrophobic compounds, for which a direct transfer mechanism is observed, regardless of the surfactant concentration in the mobile phase, the selectivity coefficient can be estimated from the ratio of two stationary-micellar partition coefficients.

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REFERENCES

1. W. L. Hinze, D. W. Armstrong, Eds., **Ordered Media in Chemical Separations**, ACS Symposium Series, Vol. 342. Washington, 1987.

2. D. W. Armstrong, F. Nome, Anal. Chem., **53**, 1662 (1981).

3. M. Arunyanart, L. Y. Cline Love, Anal. Chem., **56**, 1557 (1984).

4. A. Berthod, I. Girard, C. Gonnet, Anal. Chem., **58**, 1359 (1986).

5. M. L. Marina, S. Vera, A. R. Rodríguez, Chromatographia, **28**, 379 (1989).

6. P. Yarmchuk, R. Weinberger, R. F. Hirsch, L. J. Cline Love, Anal. Chem., **54**, 2233 (1982).

7. E. Pramauro, E. Pelizzetti, Anal. Chim. Acta, **154**, 153 (1983).

8. E. Pramauro, G. Saini, E. Pelizzetti, Anal. Chim. Acta, **166**, 166 (1984).

9. M. G. Khaledi, E. Peuler, J. Ngeh-Ngwainbi, Anal. Chem., **59**, 2738 (1987).

10. J. P. Foley, Anal. Chim. Acta, **231**, 237 (1990).

11. M. A. García, S. Vera, M. L. Marina, Chromatographia, **32**, 148 (1991).

12. M. L. Marina, M. A. García, J. Liq. Chromatogr., **17**, 957 (1994).

13. M. G. Khaledi, Anal. Chem., **60**, 876 (1988).

14. M. G. Khaledi, J. K. Strasters, A. H. Rodgers, E. D. Breyer, Anal. Chem., **62**, 130 (1990).

15. J. K. Strasters, E. D. Breyer, A. H. Rodgers, M. G. Khaledi, J. Chromatogr., **511**, 17 (1990).

16. J. K. Strasters, S. T. Kim, M. G. Khaledi, J. Chromatogr., **586**, 221 (1991).

17. A. S. Kord, M. G. Khaledi, Anal. Chem., **64**, 1894 (1992).

18. A. S. Kord, M. G. Khaledi, Anal. Chem., **64**, 1901 (1992).

19. L. S. Madamba-Tan, J. K. Strasters, M. G. Khaledi, J. Chromatogr. A, **683**, 321 (1994).

20. L. S. Madamba-Tan, J. K. Strasters, M. G. Khaledi, J. Chromatogr. A, **683**, 335 (1994).

21. J. G. Dorsey, M. T. De Echegaray, J. S. Landy, Anal. Chem., **55**, 924 (1983).

22. P. Yarmchuck, R. Weinberger, R. F. Hirsch, L. J. Cline Love, J. Chromatogr., **283**, 47 (1984).

23. A. Berthod, A. Roussel, J. Chromatogr., **449**, 349 (1988).

24. M. F. Borgerding, W. L. Hinze, Anal. Chem., **57**, 2183 (1985).

25. R. Bailey, R. M. Cassidy, Anal. Chem., **64**, 2277 (1992).

26. S. Yang, M. G. Khaledi, Anal. Chim. Acta, **294**, 135 (1994).

27. B. K. Lavine, S. Hendayana, J. Tetreault, Anal. Chem., **66**, 3458 (1994).

28. B. K. Lavine, W. T. Cooper, Y. He, S. Hendayana, J. H. Han, J. Tetreault, J. Colloid and Inter. Sci., **165**, 497 (1994).

29. J. M. Saz , M. L. Marina, J. Chromatogr. A, **687**, 1 (1994).

30. M. A. García, M. L. Marina, J. Chromatogr. A, **687**, 233 (1994).

31. M. A. García, O. Jiménez, M. L. Marina, J. Chromatogr. A, **675**, 1 (1994)

32. J. C. Miller, J. N. Miller, **Statistics for Analytical Chemistry**, John Wiley, New York, 1988.

33. I. V. Berezin, K. Martiner, A. K. Yatsimirskii, Russ. Chem. Rev., Engl. Transl., **42**, 787 (1973).

34. I. Benito, M. A. García, C. Monge, J. M. Saz, M. L. Marina, submitted.

35. M. F. Borgerding, F. H. Quina, W. L. Hinze, J. Bowermester, H. M. McNair, Anal. Chem., **60**, 2520 (1988).

36. M. A. García, S. Vera, M. Bombín, M. L. Marina, J. Chromatogr., **646**, 297 (1993).

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ANALYSIS OF CUSCOHYGRINE IN COCA LEAVES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A novel high-performance liquid chromatographic (HPLC) method is described for the determination of the alkaloid cuscohygrine [1,3-bis(1-methyl-2-pyrrolidinyl)-2-propanone] in air-dried leaves of *Erythroxylum coca* var. *coca*. The analysis was performed on a weak cation exchange HPLC column using a mobile phase consisting of MeOH: 0.05 M KH_2PO_4, pH 7 (75:25, v/v) and with UV detection at 220 nm. Cuscohygrine content was determined as 0.21-0.23% in *E. coca* leaves.

INTRODUCTION

The alkaloid cuscohygrine [1,3-bis-(1-methyl-2-pyrrolidinyl)-2-propanone] is found in a variety of plant species and its biosynthetic pathway has been shown to be similar to tropane alkaloids.[1] Alkaloid biosynthesis has become an area of increasing pharmaceutical interest because of the need to find newer and more efficient drugs in plants.

In an earlier article, we reported a high-performance liquid chromatographic (HPLC) method for the determination of hygrine in *Erythroxylum* leaf extracts using a strong cation exchange (SCX) column.[2] Cuscohygrine could not be satisfactorily resolved on this SCX column. As part of our continued interest in using HPLC for the analysis of alkaloids in *Erythroxylum spp.*, the present article describes a new HPLC method for separating cuscohygrine on a weak cation exchange (WCX) column.

EXPERIMENTAL SECTION

Chemicals

Methanol, ethanol and chloroform of HPLC grade were purchased from EM Science (Gibbstown, NJ). All other chemicals were of reagent grade or better. Water used to prepare solutions and mobile phases was initially deionized and was subsequently run through a HP Model 661A water purifier (Hewlett-Packard Co., Avondale, PA).

Cuscohygrine Synthesis

Cuscohygrine was synthesized by the same procedure as reported for hygrine in an earlier paper.[2] N-Methyl pyrrolidone (1.0 g) (Aldrich Chem. Co., Milwaukee, WI), dissolved in ethyl ether, was partially reduced to the aminoaldehyde by refluxing with $LiAlH_4$. The ether in the mixture was removed by rotary evaporation. Acetone dicarboxylic acid (1.0 g) (Aldrich Chem. Co.), dissolved in 0.1 M NaH_2PO_4, was added to the mixture. Cuscohygrine was extracted with chloroform and was then separated by vacuum distillation (10 mm) with the temperature at 85-90°C. GC/MS (mass spectrometric) analysis of the distillate revealed that cuscohygrine (m/z 224) was one of several alkaloids produced in this synthesis. After purifying the distillate by normal column chromatography using acidic alumina, a yield of 10% was obtained for cuscohygrine, density $\approx$ 1.0 g/mL.

Standard Solutions

Purified cuscohygrine (50 μL) was dissolved in 50 mL of methanol to give a standard solution of concentration 1.0 mg/mL. Standards ranging in concentrations from 0.5 to 0.05 mg/mL were prepared by serial dilutions using methanol. These standards were stored in amber vials at 0°C.

HPLC Analysis

A Model 8800 ternary gradient HPLC pump (Spectra-Physics, San Jose, CA) was used with a Model 7125 Rheodyne valve (Cotati, CA) fitted with a 5-μL loop. Cuscohygrine was separated on an Synchropak CM100 weak cation exchange (WCX) column (10.0 cm x 4.6 mm i.d., 5 μm; SynChrom, Inc., Lafayette, IN). The WCX column was used without a guard column. The mobile phase consisted of methanol: 0.05 M KH_2PO_4, pH 7.0 (75:25, v/v) delivered isocratically at 1.2 mL/min resulting in a column head pressure of about 1250 psi. Detection was made with a Model UV2000 dual wavelength detector (Thermo Separation Products, Fremont, CA) operated at 220 nm (0.05 AUFS).

Calibration Curve

The area counts of the individual peaks and the corresponding concentrations were used to construct the standard curve for cuscohygrine. The curve followed Beer's law in the range 0.1 to 1.0 mg/mL.

Extraction Procedure

Cuscohygrine was extracted from coca leaves, which were collected from plants grown under greenhouse conditions, using two separate procedures that are designated as Methods A and B. In Method A, air-dried leaves (1.0-4.0 g) were crushed by hand and refluxed in 95% ethanol (50-200 mL) at 70°C for 30 min. The extract was passed through filter paper. The solvent was removed by rotary evaporation at 60°C under vacuum (2-10 mm). The residue was re-dissolved in 50 mL of chloroform and then transferred to a separatory funnel.

The chloroform extract was shaken separately with two 25-mL volumes of 1.5% citric acid in water (w/v) which were then combined in a beaker containing a magnetic stirring bar. The aqueous layer, which was slowly stirred, was adjusted to pH 5.5 using powdered $NaHCO_3$ and was subsequently shaken with two 25-mL aliquots of chloroform. This partition step assisted in removing cocaine and other interfering alkaloids from the aqueous phase. The aqueous layer was then adjusted to pH 8.8 with 10% NH_4OH in water. Cuscohygrine was partitioned into chloroform by mixing the aqueous layer with two 25 mL volumes of chloroform in a separatory funnel. The chloroform layer was collected in an erlenmeyer flask over anhydrous Na_2SO_4 in order to remove any traces of water. The chloroform layer was then transferred to a round-bottomed flask and was subsequently reduced to a volume of 0.5 - 1.0 mL on the rotary evaporator. The extract was diluted to 8 mL with methanol for HPLC analysis.

In Method B, powdered leaf tissue (1-4 g), which was prepared by grinding the dry leaves in a Wiley mill, was combined with 50-100 mL of 95% ethanol and then was stirred for 20 min at room temperature. The extract was passed through filter paper and was handled thereafter as in Method A.

Fortified Samples

To 25-mL subsamples of a crude *E. coca* leaf extract, aliquots of a working stock solution of cuscohygrine (8 mg/mL) were added to give 1.0, 5.0, and 10.0 mg of cuscohygrine per subsample. A minimum of two replicates were made of each fortification level.

The crude leaf extract was prepared as follows: refluxed 10.0 g of dry *E. coca* leaves with 400 mL of 95% ethanol for 30 min; filtered the extract; made up the final volume of the extract to 600 mL. The mean cuscohygrine content found in the 25 mL subsamples was 0.4 mg using Methods A and B. The recovery (%) of cuscohygrine (Cusco.) was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{Amt. Cusco. (mg) Found - 0.4 mg in Subsample}}{\text{Amt. Cusco. (mg) Added}} \times 100\%$$

RESULTS AND DISCUSSION

HPLC Analysis

The chromatograms in Figure 1 show the resolution of cuscohygrine in a standard solution of hygrine (0.5 mg/mL), on (A) the SCX column and (B) the WCX column. The retention time for cuscohygrine on the WCX column was about 4.5 min at a flow rate of 1.2 ml/min. The calculated number of theoretical plates (N) was 733 plates/m. The high pH of the mobile phase, which was primarily due to the phosphate solution (pH 7), appeared to have adversely affected the WCX column by causing occasional ghost peaks and excessive baseline drift.

The calibration curve for cuscohygrine (not shown) was fitted by the regression equation $f(x) = 6.52x + 0.28$, with a coefficient of regression (r^2) of 0.99. The detection limit (signal/noise = 3) for cuscohygrine was determined as 0.05 mg/mL, or 250 ng/injection at the 0.05 AUFS sensitivity level.

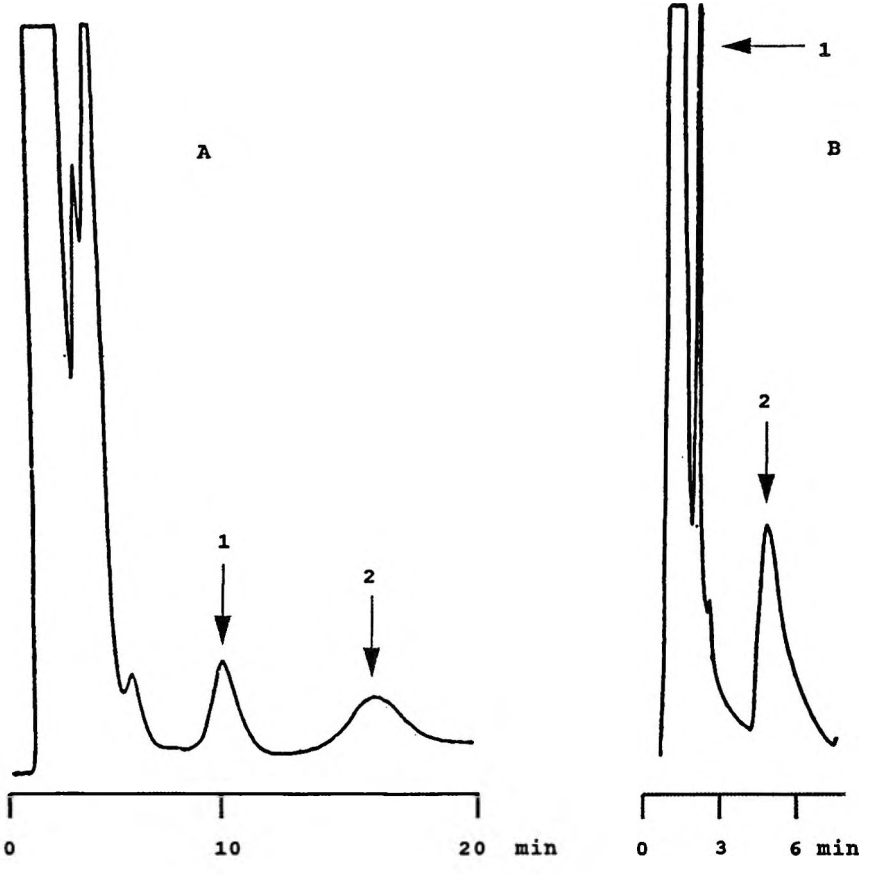

Figure 1. Chromatograms obtained from a standard solution of cuscohygrine (0.5 mg/mL) separated on (A) Column #1, the strong cation exchange (SCX) column and (B) Column #2, the weak cation exchange (WCX) column. Peaks 1 and 2 are hygrine and cuscohygrine, respectively.

Analysis of Leaves

The mean recoveries of cuscohygrine were 38.11 ± 0.18% and 64.4 ± 0.18% from fortified *E. coca* leaf extracts using Methods A and B (Table 1), respectively. The precision was similar for the Methods A and B with C.V.'s of 6.7% and 5.6%, respectively. The smaller recovery of cuscohygrine and the lower precision in Method A are attributed to the refluxing heat, which presumably caused hydrolysis of cuscohygrine in the ethanolic solution. In a

similar study involving the reflux of coca leaves in ethanolic solutions,[3] the recovery of cocaine and the precision of the method suffered significantly by longer refluxing times (i.e., 15 min or more), resulting in the hydrolysis of the alkaloid.

Table 1

Recovery (%) of Cuscohygrine from Fortified Coca Extracts[a]

Cuscohygrine (mg) Added		Recovery[b] (%)	±S.D.	C.V.%
		Method A		
1.0		24.5	0.02	6.1
5.0		50.0	0.08	3.2
10.0		39.8	0.43	10.8
	Means	38.1	0.18	6.7
		Method B		
1.0		62.0	0.04	6.4
5.0		63.0	0.01	3.03
10.0		68.3	0.40	7.23
	Means	64.4	0.18	5.57

[a] Cuscohygrine was added to 25 mL aliquots of a working stock extract which was prepared by refluxing 10.0 g of air-dried *E. coca* leaves in 600 mL of 95% ethanol. [b] Results are the mean of two to four replicates (n), standard deviation, and the coefficient of variation.

Cuscohygrine extraction required about 80 min per sample using Method A, whereas Method B required only 60 min.

The mean cuscohygrine content in unfortified *E. coca* leaves was 0.23 ± 0.03% and 0.21 ± 0.02% for Methods A and B (Table 2), respectively. There was no difference in the amounts of cuscohygrine found in unfortified samples using methods A and B as compared to the fortified samples (Table 1). Other plant components, such as lipids, amino acids, proteins, and other alkaloids, presumably inhibit the rate of hydrolysis of cuscohygrine, resulting in smaller

losses of cuscohygrine in the unfortified leaf extract. In fortified samples, there are fewer components to protect cuscohygrine from undergoing hydrolysis during reflux in ethanolic solution. The cuscohygrine content of 0.21-0.23% determined here by HPLC was slightly smaller than the 0.31% value reported for the capillary gas chromatographic (CGC) method using greenhouse-cultivated *E. coca* leaves.[4]

Table 2

Cuscohygrine Content of Air-Dried *E. Coca* Leaves

Average Dry Weight		Cuscohygrine[a] Content (%)	±S.D.	C.V.%
		Method A		
2.0 g		0.22	0.03	13.6
4.0 g		0.24	0.03	12.5
	Means	0.23	0.03	13.1
		Method B		
2.0 g		0.21	0.02	9.5
4.0 g		0.20	0.02	3.0
	Means	0.21	0.02	6.3

[a] Results are the mean of two to four replicates (n), standard deviation, and the coefficient of variation.

In conclusion, the new HPLC method described here is a simple and efficient technique for analyzing cuscohygrine and provides a reliable alternative to the capillary gas chromatographic method. In addition, the extraction procedures presented in this report allow for the specific isolation and separation of cuscohygrine and hygrine from the tropane alkaloids, e.g., cocaine and cinnamoyl-cocaine, in *E. coca* extracts.

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Mention of companies or commercial products does not imply recommendation or endorsement by the United States Department of Agriculture over others that are not mentioned.

REFERENCES

1. M. L. Newquist, T. W. Abraham, T. W., E. Leete, E., "Biosynthetic Incorporation of Ethyl (RS) [2,13-^{13}C,3-^{14}-C]-4-(1-Methyl-2-pyrrolidinyl)-3-oxobutanoate into cuscohygrine in *Erythroylum coca*," Phytochem., **46**, 254 (1993).

2. R. L. Glass, "Analysis of Hygrine in Coca Leaves using a Novel High-Performance Liquid Chromatographic Method," J. Liq. Chromatogr., **18**, 2877 (1995).

3. C. E. Turner, C. Y. Ma, M. A. Elsohly, "Constituents in Erythroxylum Coca. I: Gas Chromatographic Analysis of Cocaine from Three Locations in Peru," Bull. Narcotics **31**, 71 (1979).

4. J. M. Moore, J. F. Casale, R. F. X. Klein, D. A. Cooper, J. Lydon, "Determination and In-Depth Chromatographic Analyses of Alkaloids in South American and Greenhouse-Cultivated Coca Leaves," J. Chromatogr. A, **659**, 163 (1994).

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ANALYSIS OF CATIONIC SURFACTANTS IN HOUSEHOLD PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH NITROGEN CHEMILUMINESCENCE DETECTION

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ABSTRACT

For compounds containing less than 10% (wt./wt.) nitrogen, the sensivity of nitrogen chemiluminescence detection is limited to concentrations of greater than 0.1 mg/mL under typical operating conditions. For determinations of nitrogen containing surfactants in household products, such sensitivity is not prohibitive due to the high levels of surfactant added to the formulations and is potentially offset by the advantage of the detector's specificity towards nitrogen containing compounds. Lauryl/myristyl monoethanolamide is detected without interfence in a variety of dishwashing liquids and n-methyl glucosamides are detected in liquid laundry detergents. Replicate injections of a single dishwashing liquid preparation indicate a quantitative

reproducibility of 8% rsd for lauryl/myristyl monoethanolamide. Performance comparable to HPLC with UV detection has been achieved for methyl neodecanamide in an all purpose cleaner.

INTRODUCTION

Lauryl/myristyl monoethanolamide (LMMEA), methyl neodecanamide (MNDA) and n-methyl glucosamides are used in a variety of household products, along with quarternary ammonium compounds, ethoxylated alcohol sulfates, alkyl benzene sulfonates and other anionic, cationic, amphoteric and nonionic surfactants. The use of HPLC for the analysis of cationic surfactants was recently reviewed[1] and it was noted that for the analysis of non-aromatic surfactants, refractive index detection is used most frequently. Due to its lack of specificity, such an approach is not ideally suited for the analysis of LMMEA, MNDA or glucosamides in the presence of compounds with similar hydrophobicity, especially when it is desirable that the separation method is applicable to multiple formulations which may vary considerably in composition. To address this issue, various other approaches to the analysis of nitrogen containing surfactants, including derivatization[2-3] and post-column reaction procedures[4] have been developed. Evaporative light scattering detection has been used to allow for the use of solvent gradients.[5]

Nitrogen chemiluminescence detection (NCD),[6-8] is based on the pyrolysis of chemically bound nitrogen compounds. Specifically, column effluent is swept into a pyrolysis furnace by streams of oxygen and helium, and nitrogen containing compounds are converted to nitric oxide. The effluent is subsequently swept into a splitting tube where it is divided between a heated membrane dryer and waste.

The part of the effluent which enters the membrane dryer is pulled by vacuum into a reaction chamber, where nitric oxide reacts with ozone to form nitrogen dioxide in the excited state. Decay to the ground state, causes light emission which is measured using a photomultiplier tube and provides the basis for quantitation. An ozone generator (using oxygen as the feed gas) is used to produce the necessary flow of ozone into the reaction chamber.

Since the detector is specific to nitrogen containing compounds, it should be ideally suited for the analysis of cationic surfactants and structurally related compounds. This work describes the applicability of the detector to the analysis of LMMEA in dishwashing liquids, n-methyl glucosamides in liquid laundry detergent and MNDA in all purpose cleaner (APC), as well as the influence of detector operating variables on performance.

MATERIALS AND METHODS

Instrumentation and Methods

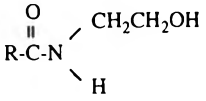
An Antek Model 7000 HPLC CLND detector (Antek Instruments, Inc., Houston, TX) was connected to a Gast DOA oil-less diaphragm vacuum pump (Gast Manufacturing Corp., Benton Harbor, MI). The CLND photomultiplier was set at 700 V for all analyses and the ozone generator to a temperature of -15°C, although this temperature was never actually achieved. The actual temperature averaged approximately -4°C. The ozone flow, the pyrolysis oxygen flow and the helium flow were set to 1.8, 3.0 and 5.5 (arbitrary units), respectively, for all analyses. For LMMEA and glucosamides, the pyrolysis temperature was 1100°C and the membrane temperature was 125°C; for MNDA the pyrolysis and membrane temperatures were set to 1050°C and 97°C, respectively.

For LMMEA analyses, chromatography was performed using a Waters model 590 pump (Waters, Milford, MA), a Rheodyne model 7520 injection valve with a 1 µL internal volume (Rheodyne Inc., Cotati, CA), and a 150 x 1mm Inertsil ODS(2) column (Phenomenex, Torrance, CA). The mobile phase was 80/20 (v/v) methanol/water at a flow rate of 0.0625 mL/min. (methanol was obtained from J. T. Baker, Phillipsburg, NJ; 15 Mohm-cm water was obtained from a Milli-Q Reagent Water System (Millipore Corp., Milford MA)). Data were collected using PE Nelson Access Chrom. Software (Perkin-Elmer, Norwalk, CT). Similar conditions were used for glucosamides except that injections were performed using a Rheodyne model 7125 valve with a 10 µL loop and a mobile phase of 75/25 (v/v) methanol water. MNDA analyses were conducted using flow injection analysis (no column). To reduce pump pulses an Upchurch Scientific 500psi BPR (Upchurch Scientific, Oak Harbor, WA) was placed between the pump and the 1 µL injection valve. Methanol, at a flow rate of 0.0625 mL/min, was used to transfer the sample from the injector to the detector.

Sample Preparation

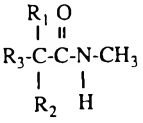
All standards and samples were obtained in-house. For LMMEA, standards were prepared to operating concentrations of 0.5 - 2 mg/mL in 80/20 (v/v) methanol/water. Dishwashing liquid samples were prepared by dissolving 5g / 50 mL. The glucosamide standard and samples were prepared by dissolving 50mg of lauryl/myristyl n-methyl glucosamide in 50 mL and 2.5g of liquid laundry detergent in 100 mL of 70/30 (v/v) methanol/water. MNDA standards were prepared in methanol to operating concentrations of 0.1 - 1.0

LMMEA (6.03% N)



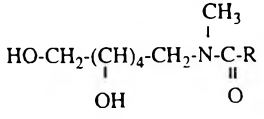
$R = C_{12}H_{25}$ or $C_{14}H_{29}$

MNDA (8.00% N)



$R_1 + R_2 + R_3 = C_8H_{19}$

GLUCOSAMIDE (3.46% N)



$R = C_{12}H_{25}$ or $C_{14}H_{29}$

Figure 1. Chemical structures and % nitrogen of LMMEA, MNDA and glucosamides.

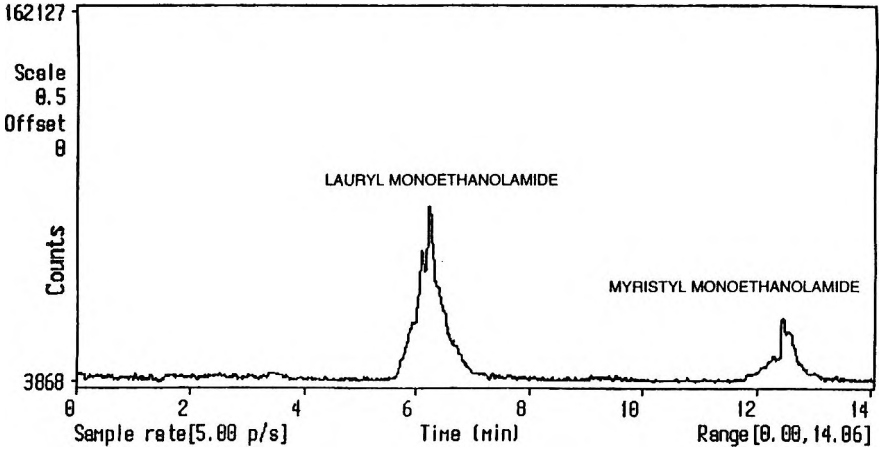


Figure 2. Chromatogram of an LMMEA standard. Pyrolysis temperature = 1050°C, membrane temperature = 97 C. For additional conditions see text.

mg/mL. Samples were prepared by dissolving 2.5g of APC in 50 mL of methanol. All samples were filtered prior to injection using 0.45 μm Autovial syringeless filters (Whatmann, Clifton, NJ).

RESULTS AND DISCUSSION

A primary advantage of nitrogen chemiluminescence detection is selectivity, potentially making it possible to analyze for nitrogen containing compounds in the presence of coeluting compounds. However, several limitations exist: The effluent entering the detector must be less than 200 μL/min., which requires the use of microbore columns or the use of post-column splitters; acetonitrile cannot be used as a mobile phase constituent; and the sensitivity of the detector is limited to approximately 0.4ng of nitrogen under favorable chromatographic conditions.[7]

Surfactants are typically added to cleaning formulations at fairly high levels (>0.25% wt/wt). As a result, the sensitivity should not be a limiting factor despite the low nitrogen content of the compounds of interest (Figure 1). The substitution of methanol for acetonitrile in reverse phase HPLC changes selectivity and increases solvent viscosity, but by virtue of the better specificity of the detector, the increased back pressure generated using methanol is offset by the lower separation efficiency required. For simpler separations, it is possible to use shorter columns or columns packed with larger particles to separate the analyte from the matrix.

To evaluate NCD for the analysis of LMMEA in dishwashing liquids, manufacturer recommended settings[8] were used as a starting point for method development. Specifically, the pyrolysis tube temperature was set at 1050°C, the membrane dryer temperature at 97°C and the ozone flow, the pyrolysis oxygen flow and the helium flow to 1.8, 3.0 and 5.5 (arbitrary units), respectively. Under these conditions (Figure 2) poor peak shapes were observed. Variation in the membrane dryer temperature was insignificant over the range studied (97 -150°C), and variations in the gas flows either had no effect or decreased the signal to noise ratio. The effect of increasing the pyrolysis temperature to 1100°C was, however, pronounced. Using the latter conditions, both the peak shapes and the sensitivity are acceptable (Figure 3). While the concentration of LMMEA in the standard shown in Figure 3 is 2 mg/mL, the mass of nitrogen injected is approximately 120 ng.

The conditions of Figure 3, were used for the analysis of several different dishwashing liquids. Despite variations in the formulations, both LMMEA peaks were resolved from the matrix, showing the applicability of the method for qualitative analysis. A typical chromatogram is shown in Figure 4. To investigate the reproducibility of the method, 6 replicate injections of a prepared sample were performed and an rsd of 8% was observed. Since the influence of LMMEA concentration on product efficacy is critical, it is important to reduce this variability.

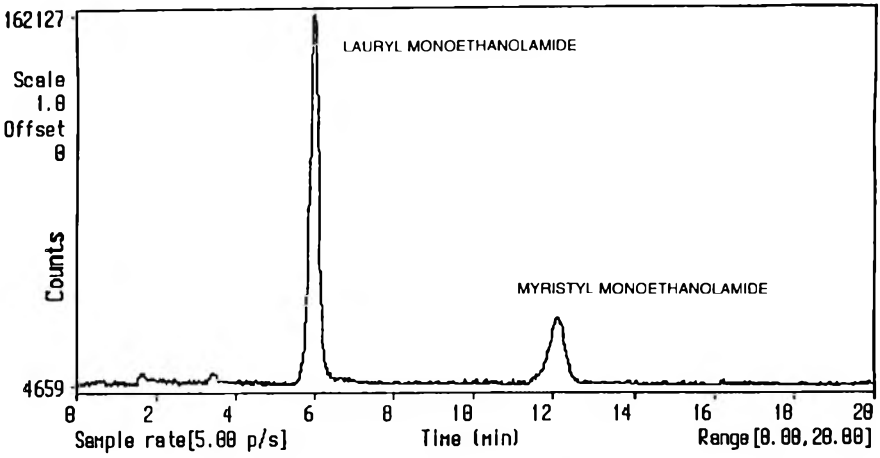


Figure 3. Chromatogram of an LMMEA standard. Pyrolysis temperature = 1100°C, membrane temperature = 125 C. For additional conditions see text.

Table 1

Comparison of Flow-Injection - NCD with HPLC-UV for the Analysis of MNDA in APC

	HPLC-UV	FI-NCD
RSD of 6 determinations	1.5%	2.0%
Analysis of 0.25% sample	0.260%	0.243%
Analysis of 0.50% sample	0.500%	0.480%
Analysis of 0.75% sample	0.780%	0.770%
Regression (r)	0.9961	0.9996

The poor reproducibility observed for LMMEA was not observed for MNDA in an all purpose cleaner using flow injection analysis. Table I, compares the results to those obtained using an HPLC method with UV detection described previously.[9] As shown, the performance of the CND method is comparable to that of the HPLC-UV method with respect to accuracy and precision.

As a result of the performance of the detector with MNDA, it was concluded that the large rsd, observed for LMMEA samples, was due to the nature of the analyte and/or the sample matrix. Notably, after the analysis of

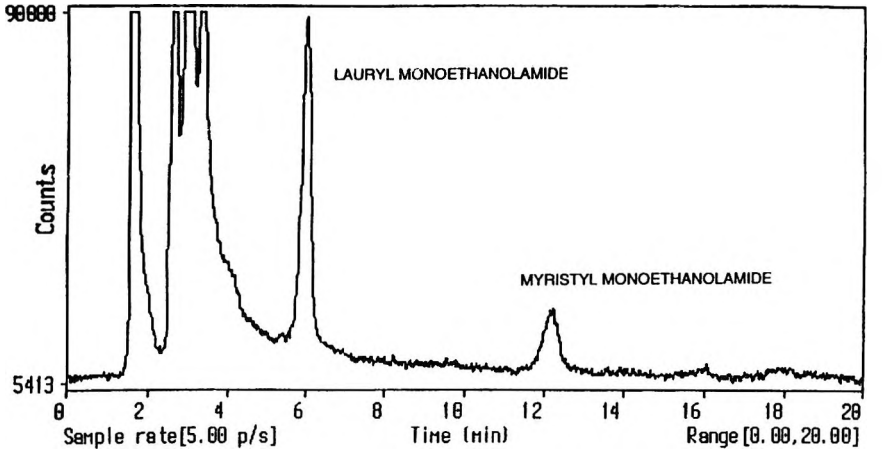


Figure 4. Chromatogram of LMMEA in a commercially available dishwashing liquid. Conditions are as for Fig. 3.

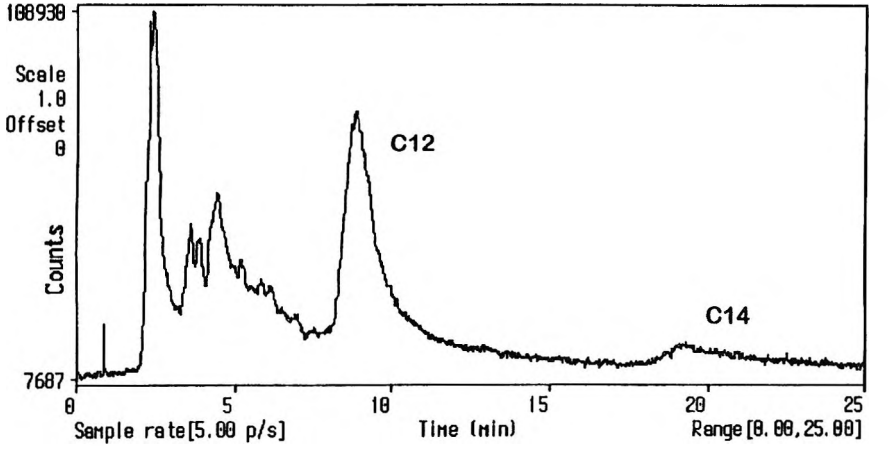


Figure 5. Chromatogram of lauryl/myristyl n-methyl glucosamide in liquid laundry detergent. For conditions see text.

several dishwashing liquid samples, it was noted that the waste outlet on the splitter, which controls the amount of material entering the membrane dryer, was partially clogged. Variability in the back pressure of the outlet should directly influence the split ratio and, therefore, the amount of analyte entering the ozone reaction chamber. Most likely, the problem is due to incomplete

pyrolysis of dishwashing liquid matrix components and could possibly be minimized by sample clean-up procedures prior to chromatography. As a more general solution, we are currently investigating means of modifying the splitter design to minimize deposition at the waste outlet.

While additional research is required to improve reproducibility and ruggedness, CND is currently useful for qualitative analysis. As an example, the detector has been used to screen liquid laundry detergents for glucosamides (Figure 5). In addition, several products previously screened for LMMEA content using refractive index detection and retention time for identification, have been shown to have provided false positives.

REFERENCES

1. B. P. McPherson, H. T. Rasmussen, "Chromatography of Cationic Surfactants: HPLC, TLC, and GLC" in **Cationic Surfactants: Analytical and Biological Evaluation,** J. Cross and E. J. Singer, eds., Marcel Dekker, Inc., New York, 1994. Chapter 10.

2. P. Jandera, H. Pechova, D. Tocksteinova, J. Churacek, Chromatographia, **16**, 275 (1982).

3. M. C. Gennaro, E. Mentasti, C. Sarzanini, V. Porta, Chromatographia, **25**, 117 (1988).

4. J. Kawase, Y. Takano, K. Tsuji, J. Chromatogr., **262**, 293 (1983).

5. A. J. Wilkes, C. Walraven, J. -M. Talbot, J. Am. Oil Chem. Soc., **69**, 609 (1992).

6. E. M. Fujinari, L. O. Courthauden, J. Chromatogr., **592**, 209 (1992).

7. E. M. Fujinari, J. D. Manes, J. Chromatogr., **676**, 113 (1994).

8. Specifications. HPLC-CLND. Antek Instruments, Inc., Houston, TX, 1992.

9. H. T. Rasmussen, S. K. Friedman, A. J. Mustilli, R. McDonough, B. P. McPherson, J. Liq. Chromatogr., **17**, 589 (1994).

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PURIFICATION OF PHOSPHATIDYLCHOLINE WITH HIGH CONTENT OF DHA FROM SQUID *ILLEX ARGENTINUS* BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

A method for purification by countercurrent chromatography (CCC) of phosphatidylcholine with high content of DHA is described. Phospholipids were extracted from squid *Illex argentinus*, characterized by high performance liquid chromatography and their fatty acids profile determined by gas chromatography. Phosphatidylcholine was primarily represented with 48.3 % (w/w) of total phospholipids and contained 39.7 % of DHA (w/w total fatty acids). The analytical separation of phosphatidylcholine, was achieved on a Kromaton II apparatus, in the systems heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) and isooctane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v), at 550 rpm and 1.5 mL/min flow rate using normal ascending and reversed descending elution modes respectively.

The fractions collected were further analysed by high performance thin layer chromatography. Phosphatidylcholine was separated from the other phospholipids of the crude extract and was recovered pure in the lower phase with 95 % yield. Purification at preparative scale was performed on a 1 L column, 550 rpm and 5 mL/min using the same chromatograph. These results prove that CCC constitutes a powerful technique for separation of phospholipids from natural lipidic extracts.

INTRODUCTION

Nutritional importance of polyunsaturated fatty acids (PUFA) has considerably increased during the past decade. In animals, linoleic acid (C18:2 ω6) and α-linolenic acid (C18:3 ω3) found in vegetable food, are metabolized into arachidonic acid (AA, C20:4 ω6) and docosahexaenoic acid (DHA, C22:6 ω3) which are constituents of cellular membranes and play essential role in neuronal and sensorial development.[1] Previous studies have shown, that dietary DHA supply during gestation and feeding is a nutritional factor important for nervous structures development in young mammals.[2]

Recently, Makrides et al.[3] in a comparative study through new-born infants, have found there is a positive correlation between DHA level in erythrocytes and maturation of visual acuity, and Leger et al.[4] have confirmed the high content of phosphatidylcholine (PC) and DHA during constitution of retina in human embryo.

Therefore, in prematures babies who can not be breast-fed or take benefit from human milk, it may be necessary to supply dietary formula with PUFA and especially DHA from fish oil or egg yolk.[5] Here in, is described the use of countercurrent chromatography (CCC) to obtain pure PC with high content of DHA from cephalopod skin, a potentially new source of PUFA.

Some previous studies[6-9] have proposed methodologies to separate various lipid classes including fatty acids esters by centrifugal partition chromatography (CPC). For example, Alvarez et al.[10] succeeded in separating PE and PC standards in heptane-ethanol solvent-system.

Recently, Bousquet et al.[11] have purified PUFA from microalgae by CCC but, no data were collected on separation of phosphatides by this technique. We present, in this paper, the separation of PC from squid phospholipids crude extract by CCC in two solvent-systems.

MATERIALS AND METHODS

Reagents

Solvents of analytical grade were from Carlo Erba (Nanterre, France) or Merck (Nogent sur Marne, France), BHT and phospholipids standards were obtained from Sigma (St Quentin fallavier, France).

Skins from *Illex argentinus* squid were kindly provided by SCA (Société commerciale de l'Adour, Bayonne - France).

Extraction of Phospholipids

Squid skins were cut in small pieces and blended in Stephan grinder at +4°C to give a fresh paste. The phospholipids were extracted directly from the paste by a method adapted from Singleton et al.[12] This method is based on the different solubilities of the various classes of lipids in cold acetone : most of the glycolipids and phosphatides, especially acidic phosphatides in salt form, are insoluble in acetone at -20°C when glycerides and other neutral lipids are soluble in the same conditions.

The solvents used for extraction contained 0.1 % w/v butylated hydroxy toluene (BHT) in order to avoid any oxidation.

In practice, the paste was agitated thorougly with 5 volumes of acetone (v/w) in batch for 2 hrs at 25 °C. The mixture was then filtered and the solids washed three times with one volume of cold acetone. The filtrate was kept at -20°C during 16 hrs to precipitate the phospholipids. The solution was then centrifuged at 2500 rpm for 10 min, acetone supernatant was removed and the pellet was dried under nitrogen.

The acetone-insoluble material contained most of the phospholipids and traces of neutral lipid, whereas, the acetone-soluble fraction contained all the neutral lipids such as glycerides, sterols, sterol esters, hydrocarbons, pigments (carotenoids) and only traces of phosphatides.[13] The crude phosphatides were dissolved in methanol, placed into a rotavory evaporator for the removal of solvent and dried under nitrogen. The crude extract was stored in chloroform at -20°C.

Total lipids Analysis

Total lipids in the crude extract were quantified by extraction with hexane in a Soxhlet apparatus. Fractionation, of the various classes of lipids (neutral lipids, glycolipids and phospholipids), was performed by adsorption chromatography on silica gel (Silica gel 60, particle size 63-200 μm, 70 - 230 mesh, Merck, Nogent sur Marne, France), using a modification of the method described by Borgstom[14] and adapted by Rouser et al.[15] and Soudant et al.[16] 300 mg silica gel in chloroform was used for 10 mg of lipid extract. Neutral lipids were eluted with 10 mL of chloroform, glycolipids with 15 mL acetone - methanol (9:1, v/v) and phospholipids were recovered with 10 mL of methanol. Each class was quantified after solvent evaporation under nitrogen and expressed as % of total lipids.

High Performance Liquid Chromatography

Phospholipids were separated by HPLC as previously described,[17,18] on a column 25 cm long, 7.5 mm internal diameter filled with Lichrosorb Si 60 (5 μm particle size, Merck, Nogent sur Marne, France) using a System Gold 126 chromatograph (Beckman, Gagny, France) equiped with a gradient pump and injection loop of 250 μL. Solvents used were solvent A: hexane, solvent B: isopropanol - chloroform (4:1, v/v), and solvent C: isopropanol - water (1:1, v/v). Crude extract (3 to 5 mg) was injected in solvent D: A - B - C (42:52:6, v/v/v). Elution was performed at room temperature with a linear gradient from 100 % solvent D to 100 % solvent E: A - B - C (32:52:16, v/v/v) in 20 min at 2.5 mL/min flow rate. Detection of the various classes of phospholipids was achieved using a light-scattering detector DDL 11 (Cunow, Cergy-Pontoise, France). Effluents were partially collected from the column to determine the fatty acids profile of each class of phospholipids.

High Performance Thin Layer Chromatography

Lipidic content of fractions collected by countercurrent chromatography was analyzed by HPTLC using an automated multiple development apparatus AMD (Camag, Muttenz, Switzerland). Phospholipids were identified on pre-coated plates (Silica gel 60 F-254, Merck, Nogent sur Marne, France), which were prewashed for 1 hour by diving in isopropanol and then dryed for 30 min at 120°C. Standards and samples application in 6 mm length bands, 4 mm apart, was performed at 4 sec/μl delivery rate by the spray-on technique using a Linomat IV (Camag, Muttenz, Switzerland). The principle of AMD has been previously described.[19,20]

In the present work, we used a 25 steps universal gradient fully automated. The gradient started with the solvent possessing the strongest elution power and in the successive runs the polarity was decreased. Between runs, the solvent previously used was eliminated from the development chamber and TLC plate was dried under nitrogen.

After chromatographic development the plates were dived for 5 seconds in a solution containing 4 mL concentrated sulphuric acid, an 0.4 g manganese chloride tetrahydrate in 120 mL water-methanol (1:1, v/v). The plates were then heated at 120°C for 30 min. Phospholipids appeared as brown zones on a white background and absorption at 550 nm was measured with a TLC Scanner II apparatus (Camag, Muttenz, Switzerland).

Each compound was identified by its Rf and compared with authentic lipid standard.

Gas Chromatography

Total phospholipids (< 5 mg) were dissolved in 5 mL ethanol containing 10% KOH. After 15 hours, released fatty acids were extracted twice with 10 mL hexane, dried under-vacuum and then methylated by refluxing in 10 mL solution HCl - methanol 3 % (v/v) for 1 hour. Fatty acids esters were extracted in hexane, dried under-vacuum and dissolved in chloroform before gas chromatography (GC) analysis.

Phospholipids collected from HPLC, were submitted to methylation with BF3 in 10 % methanolic solution at 90°C for 20 min (PE, PC, PS, PI) or 90 min (SM) according to Morrisson and Smith.[21] Fatty acids methyl esters were collected in hexane and conditioned as described above.

The fatty acids esters were analyzed by GC on a capillary gas column CPWAX 52 CB (50 m long, 0.32 mm diameter, 0.25 μm thickness stationary phase) from Chrompack (Les Ulis, France), using a GC 8000 chromatograph (Fisons, Arcueil, France) fitted with a AS 800 injector and a FID 80 detector. The carrier gas was hydrogen at pressure 1.2 bar and the temperature was programmed from 54°C (for 3 min) to 194°C at 3°C per min.

Samples were injected at 54°C and the detection was carried out at 250°C. Fatty acids esters were identified by their retention times and quantified with reference to standards.

Table 1

Phospholipidic Composition of the Crude Extract Expressed in Percentage of Total Phospholipids

Classes of Phospholipids	% of Total Phospholipids
PE	24.1
PI	17.5
PS	4.9
PC	48.3
SM	5.1

PE: phosphatidylethanolamine; PI: phosphatidylinositol;
PS: phosphatidylserine; PC phosphatidylcholine
SM: Sphingomyellin

Table 2

Fatty Acid Composition of Crude Extract Phospholipids Expressed in Percentage of Fatty Acids

Fatty Acids	Total Phospholipids	PE	PC	PS	PI	SM
Tot. Sat.	35.5	25.1	44.3	34.1	51.5	82.5
Tot. Mon.	8.2	10.0	7.5	12.6	15.4	11.0
20:4n-6	4.3	10.2	1.0	3.3	4.1	0.3
20:5n-3	16.7	34.6	5.0	12.3	20.7	1.0
22:6n-3	33.1	18.5	39.7	35.0	7.1	4.2

Tot. Sat.: Total saturated fatty acids;
Tot. Mon.: Total monounsaturated fatty acids.

Countercurrent Chromatography

The separation of PC from crude extract was carried out with a countercurrent chromatography instrument (Kromaton II SEAB, Villejuif, France). The column was 2.4 mm internal diameter, 5 m long,

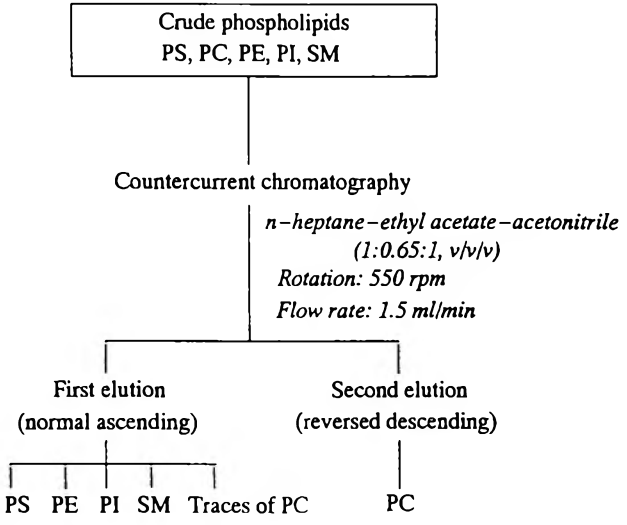

Figure 1. Diagram illustrating the elution modes and separation of phosphatidylcholine by countercurrent chromatography. PS: phosphatidylserine, PE: phosphatidyl-ethanolamine, PI: phosphatidylinositol, SM: sphingomyelin, CP: cholesterol and cholesterol esters.

polytetrafluoroethylene (PTFE) tubing wound on the original holder; its total volume was 75 mL. The rotational speed was 550 rpm.

The chromatographic system was a Kontron 420 isocratic, constant solvent-delivery pump and a fraction collector Frac 300 from Pharmacia.

The partition coefficient of phosphatidylcholine in the binary heptane-acetonitrile and isooctane-acetonitrile two-phase solvent system was favorably modified by addition of ethyl acetate. The two biphasic systems used were heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) and isooctane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) . The solvents were filtered before used.

Phosphatidylcholine was separated from the other phospholipids at a flow rate of 1.5 mL/min, using the system described as normal ascending and reversed descending elution modes respectively. Fractions were collected every 4 min and were cooled in the dark at -20°C before analysis.

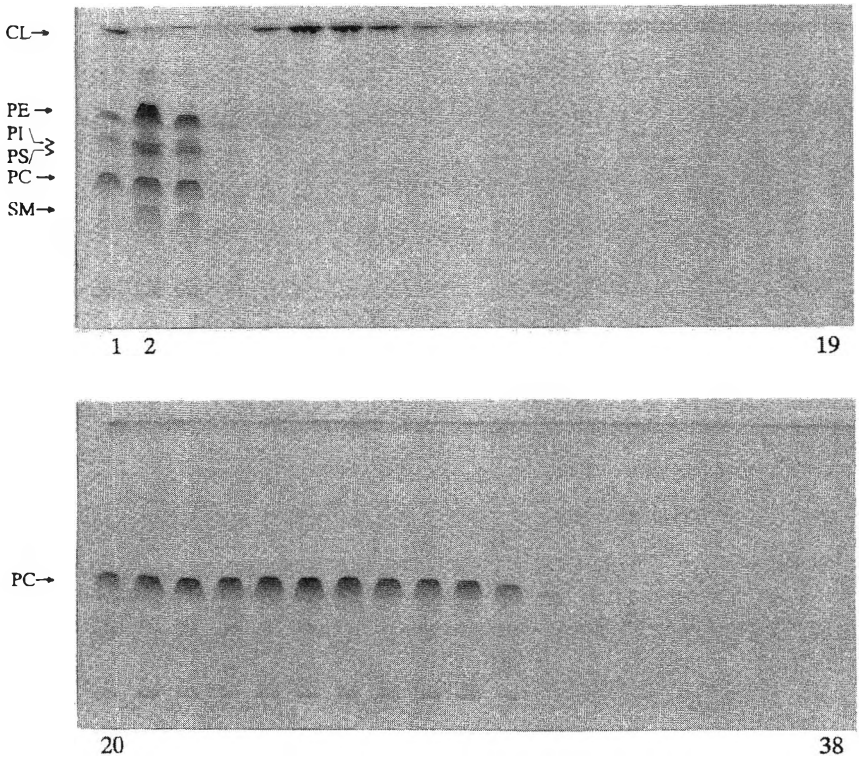

Figure 2. HPTLC analysis of fractions collected by countercurrent chromatography on Kromaton II at 550 rpm and 1.5 mL per mn in solvent system heptane - ethyl acetate - acetonitrile. Plates were developped with a 25 steps gradient in AMD apparatus and revelation of phospholipids was performed with a sulphuric acid and manganese chloride solution in water-methanol 1:1 (v/v). 1, crude extract 50 µg; 2 to 19, upper phase fractions; 20 to 38, lower phase fractions. CL, cholesterol and cholesterol esters; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin.

RESUTS AND DISCUSSION

The crude extract was obtained with 0.72 % yield from rough squid skins and contained 90.3 % of total lipids. The phospholipids represented 97.7 % (w/w) of total lipids by quantification on silica gel. Separation of the various classes of phospholipids was achieved by HPLC and showed that PC was primarily represented with 48.3 % (w/w) of total phospholipids (Table 1). The

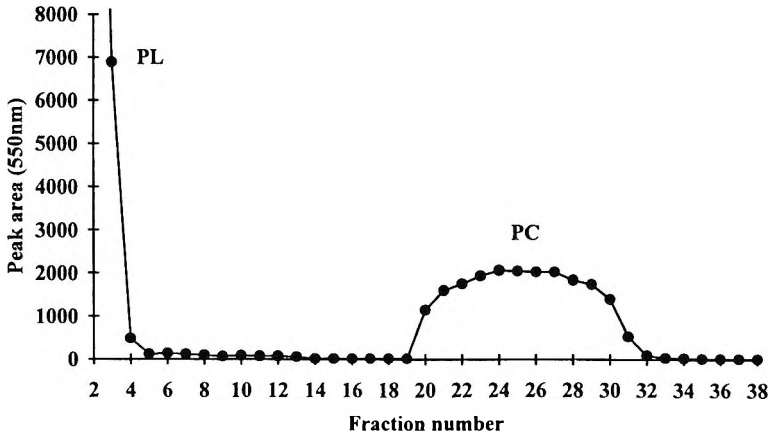

Figure 3. Absorption at 550 nm of phospholipidic content in collected fractions measured on HPTLC plates with TLC scanner II apparatus. Pure PC was collected in fractions 19 to 31 of the lower phase and separated from the other phospholipids of the crude extract found in the upper phase. 2 to 19, upper phase fractions; 20 to 38, lower phase fractions. PL, phospholipids of the crude extract; PC, pure phosphatidylcholine.

final PC content in the crude extract was then 42.6 % (w/w). The crude extract yield was increased to 1.38 % when paste was stored at -20°C for 60 days before acetone treatment, probably because of higher release of lipidic constituents from grinded skins. But, at the same time, PC content was decreased to 36.3 % (w/w total phospholipids).

The fatty acids profile obtained for each class of phospholipids showed that DHA content of PC was 39.7 % and EPA 5.0 % of total fatty acids (Table 2). High DHA content 35.0 % was also exhibited by PS and high EPA content 34.6 % by PE (respectively 4.9 % and 24.1 % of total phospholipids).

Phosphatidylcholine was purified by CCC from the crude extract in the system heptane-ethyl acetate-acetonitrile (Figure 1). Experiments were carried out dissolving 0.32 g to 0.64 g of total phosphatides in 5 mL of mobile phase and injecting into the analytic column. The upper phase (less polar phase) was used as mobile phase. The less polar phospholipids were eluted first, whereas, the most polar remained in the column.

The first three fractions contained phospholipids PE, PI, PS, SM and traces of PC as shown in Figure 2. One contaminant compound was then collected in the next fractions 5 to 12 and analysis showed it was a mixture containing 89

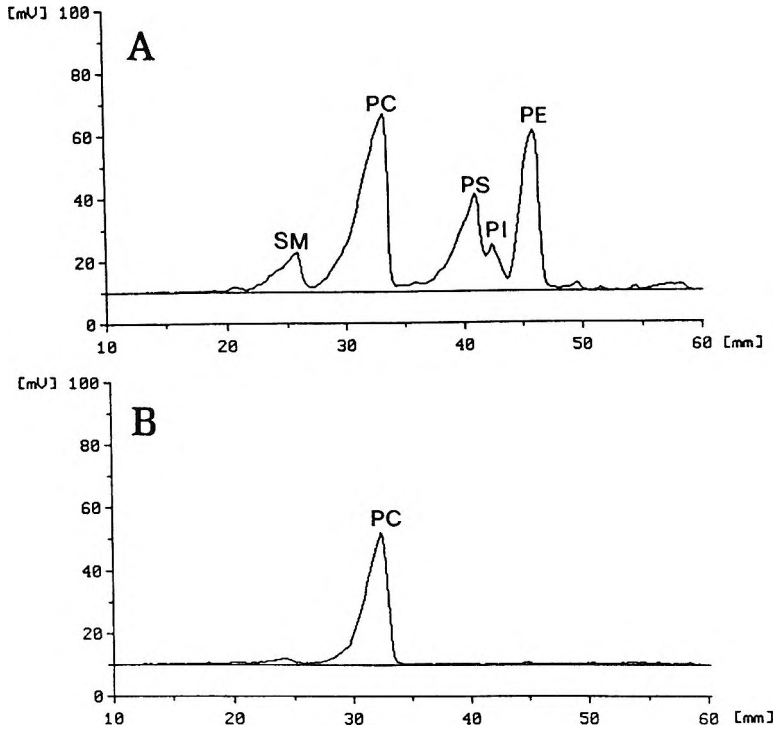

Figure 4. Detection at 550 nm of the various classes of phospholipids on HPTLC plates with TLC scanner II apparatus. A, crude extract; B, fraction containing pure phosphatidylcholine collected by CCC.

% cholesterol, 5 % cholesterol esters and little amount of PC. After running one volume of mobile phase through the column in the normal ascending mode, phosphatidylcholine was eluted in the stationary phase (lower phase) as shown in Figure 3. PC was obtained pure (Figure 4) with an estimated yield of 95 % of total injected PC. Heptane was then replaced by isooctane in the CCC solvent-system for purification of PC from crude extract. Results obtained were identical using heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) or isooctane - ethyl acetate -acetonitrile (1:0.65:1, v/v/v) solvent-system.

One main problem encountered for purification of phosphatides by partition in solvent-systems, is the possibility to induce emulsions that could remain stable. This property reduces the choice of solvents we can use for CCC experiments. The systems hexane - methanol - water (1:0.9:0.1, v/v/v) and hexane - ethanol - water (1:0.9:0.1, v/v/v), previously described by Murayama

et al[6] for purification of PUFA esters by CPC, contained water and were not suitable for separation of phosphatides by CCC. So, we started with the system heptane - acetonitrile (1:1, v/v) exempt of water described by Bousquet et al.[11] for the purification of PUFA from microalgae by CCC. We made it suitable for the separation of polar lipids, such as phosphatides, by adjusting the partition coefficient of phosphatides with ethyl acetate. The value obtained for phosphatidylcholine was 1.01 in the system heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v).

This system showed a good behavior in CCC and proved to be well adapted for separation of PC from natural phospholipids. Scale-up experiment was carried out with a 1 L preparative column on the same CCC chromatograph in the system heptane - ethyl acetate - acetonitrile (1:0.65:1, v/v/v) at a flow rate of 5 mL/min and 550 rpm and we succeeded in obtaining pure PC as described above.

The purification of phosphatidylcholine has been the subject of extensive investigations essentially by classic methods such as HPLC. In this study we demonstrate the usefulness of CCC for the same purpose.

REFERENCES

1. T. Desci, B. Koletsko, Acta Paediatr.suppl., **395**, 31-37 (1994).

2. P. Guesnet, J. M. Alessandri, Cah. Nutr. Diét., **30**, 109-116 (1995).

3. M. Makrides, K. Simmer, M. Goggin, R. A. Gibson, Pediatric Res., **33**, 425-427 (1993).

4. C. L. Leger, G. Fouret, S. Bouvier, P. Sarda, B. Descomps, OCL, **2**, 45-51(1995).

5. T. Sanders, Proc. Nut. Soc., **52**, 457-472 (1993).

6. W. Murayama, Y. Kosuge, N. Nakaya, Y. Nunogaki, K. Nunogaki, J. Cazes, H. Nunogaki, J. Liq. Chromatogr., **11**, 283-300 (1988).

7. Y. Tanaka, T. Funada, W. Murayama, Y. Kosuge, J. Jpn Oil Chem. Soc., **41**, 23-27 (1992).

8. Y. Tanaka,T. Funada, W. Murayama, Y. Kosuge, J. Jpn Oil Chem. Soc., **41**, 312-316 (1992)

9. J. Cazes, Biotechnology, **6**, 1398-1400 (1988).

10. J. G. Alvarez, J. Cazes, J. C. Touchstone, R. L. Grob, J. Liq. Chromatogr., **13**, 3603-3614 (1990).

11. O. Bousquet, N. Sellier, F. Le Goffic, Chromatographia, **39**, 40-44 (1994).

12. W. S. Singleton, M. S. Gray, M. L. Brown, J. L. White, J. Am. Oil Chem. Soc., **42**, 53- 56 (1965).

13. M. Kates, "Separation of Lipid Mixtures", in **Techniques of Lipidology**, R. H. Burdon, P. H. van Knippenberg, eds., Elvesevier, Amsterdam (2nd edition),1986, pp. 187-188.

14. B. Borgstom, Can. J. Biochem. Phys., **37**, 911-917 (1952).

15. G. Rouser, G. Kritchevsky, A. Yamamoto, **Lipid Chromatographic Analyses**, Dekker, New York (2nd edition), 1976.

16. P. Soudant, Y. Marty, J. Moal, J. F. Samain, J. Chromatogr., in press.

17. W. W. Christies, J. Lipid Res., **26**, 507-512 (1985).

18. P. Juaneda, G. Rocquelin, P. O. Astorg, Lipids, **25**, 756-759 (1990).

19. K. Burger, Z. Fresenius, Anal. Chem., **318**, 228-233 (1984).

20. F. Bonté, P. Pinguet, J. M. Chevalier, A. Meybeck, J. Chromatogr., **664**, 311-316 (1995).

21. W. Morrisson, L. Smith, J. Lipid Res., **5**, 600-608 (1964).

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FREE BILE ACID ANALYSIS BY SUPERCRITICAL FLUID CHROMATOGRAPHY AND EVAPORATIVE LIGHT SCATTERING DETECTION

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ABSTRACT

Packed column supercritical fluid chromatography of free bile acids is described. Improvement of the separation of isomeric acids is carried out on diol-bonded silica using methanol or isopropanol as modifier of carbon dioxide. These poor UV absorbing analytes were detected by evaporative light scattering detection showing the potentiality of the SFC-ELSD coupling.

INTRODUCTION

Bile acids are steroids with 24 carbon atoms biosynthesized from cholesterol and which occur in some biological fluids both in the free and conjugate forms. They include primary bile acids (i.e. cholic and chenodeoxycholic acids) and secondary bile acids by deshydroxylation (i.e. deoxycholic acid and lithocholic acid). The complete simultaneous separation of dihydroxylated acids is complicated by the isomeric forms due to the hydroxyl groups.

Bile acids are present in stools, mainly as free bile acids. They can also be used as therapeutic agents for the dissolution of cholesterol gallstones and in the therapy of bile reflux gastritis.

Usually the determination of free bile acids is based on reverse phase liquid chromatography with buffer due to their pKa value (about 6). Selectivity is highly dependent on the composition of the mobile phase: the nature of the buffer, the ionic strength and the pH value. Thus the limitations of this method are:

- low sensitivity in the detection by UV absorption (amount about 200 nmole).[1]
- gradient elution involves a severe drift of baseline[1] at the low wavelength used (210 nm).

A. Roda[2,3] proposed evaporative light scattering detection (ELSD) in place of UV detection in liquid chromatography. The mobile phase is nebulized by a gas stream and the aerosol droplets are carried through a heated tube to evaporate the mobile phase. The remaining particles of nonvolatile solutes pass through a light beam and the light is scattered and collected by a photomultiplier or photodiode, causing a chromatographic signal which depends on the amount of solute, but not on its nature. The limit of detection of bile acids is about 5 nmole i.e. 30-50 times lower than with UV detection[2] and permits a mass detection. However, the ammonium acetate required to give an ionic strength, needs a sufficiently high temperature to ensure complete vaporization of the mobile phase and to avoid baseline noise . The ELSD used in this work,[2,3] requires a heated temperature of 130°C which is high enough for thermodegradation with thermolabile products.

Supercritical Fluid Chromatography (SFC) using packed column offers the advantage of a simple mobile phase (CO_2-organic modifier). The polarity of this mobile phase can be easily modified by varying the modifier content and/or by increasing the column pressure, because density and thus solvent power increases highly with an increase in pressure. This method has been used for bile acids[4] and affords an analysis time faster than that obtained by the reverse phase.[5] In this work,[4] UV detection at low wavelength and the use of methanol lead to a poor sensitivity. Moreover, only five bile acids are separated and the mechanism remains somewhat unclear.

We have proposed a new ELSD in which the temperature of the heated tube is low, thus avoiding a degradation of thermolabile compounds.[6] We also propose coupling this detector with SFC.[7] Hence, this technique appears to be a universal detector for packed column SFC.

The aim of this work is to show that the separation of seven naturally occurring free bile acids can be achieved using packed column SFC. Different parameters are studied to increase the selectivity of the separation and the sensitivity of the detection.

EXPERIMENTAL

The SFC apparatus consisted of the Series SF3 supercritical fluid chromatographic system (Gilson, Villiers-le-Bel, France): a Model 305 piston pump (main pump) delivers the CO_2. Polar modifier was added by a pump Model 302 (slave pump) and both solvents were mixed in a Model 811 C mixer. The head of the Model 305 pump was cooled with a refrigerated bath.

Supercritical or subcritical conditions were maintained by a pressure regulator valve (Model 821) set at the column outlet and before a fused silica capillary, which acts as a restrictor to maintain mobile phase in a critical state before detection. This restrictor is set in the heated specific interface of the ELSD for SFC (Sedex Model 55, Touzart et Matignon, Vitry-sur-Seine, France) which allows both expansion and nebulization of the supercritical mobile phase before its vaporization in the heated tube. Ice formation at the outlet of the restrictor and condensation of the organic modifier in the interface of detector, are avoided thanks to a make-up gas.

The column was thermostated at 50°C in a Croco Cil oven (CILCluzeau, Sainte-Foy la Grande, France). Samples were injected onto the column via a Rheodyne 7125 valve fitted with a 25 µL sample loop. Chromatograms were recorded on a Shimadzu Model CR5A integrator (Touzart et Matignon, Vitry-sur-Seine, France)

Columns: Lichrosorb Diol (5 µm (150x4.6 mm) Interchrom (Interchim, Montluçon, France), Zorbax NH_2 and TMS (Dupont, Wilmington, DE, USA), Pecospher HS-3 Silica(Perkin-Elmer, Norwalk, Connecticut, USA). Carbon dioxide was purchased from Air Liquide (Paris, France) and methanol and isopropanol were of HPLC grade from Prolabo (Paris, France).

Bile acids were of analytical grade: lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), hyocholic acid (HCA), cholic acid (CA). Fig. 1 illustrates two analytes: a primary acid (CA) and a secondary acid (DCA). Analytes were dissolved in methanol/dichloromethane 50:50 mixture at the concentration of 200 ppm.

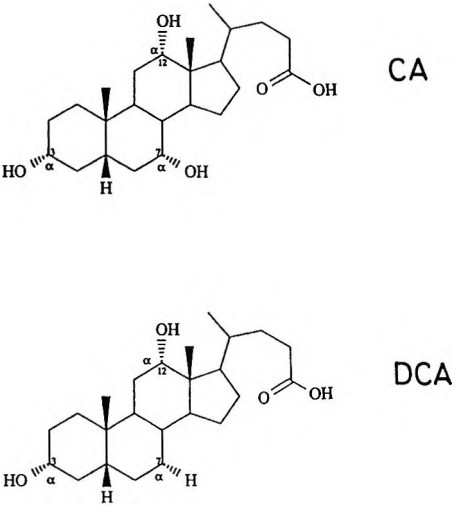

Figure 1. Scheme of bile acids : CA = cholic acid, DCA = deoxycholic acid.

RESULTS AND DISCUSSION

Several columns (Zorbax NH_2, Zorbax TMS, Pecospher Silica and Lichrosorb Diol) were studied with CO_2-methanol as mobile phase. Only Lichrosorb Diol affords a good shape of peaks, selectivity and easy use. Therefore, the following study gives results on this stationary phase. Analyses were carried out at the 3 mL/min flow-rate. This value is nearly the optimum value obtained by[8]

$$F_{opt}\,(mL/min) = 1.25 \times dc^2(mm^2)/dp\,(\mu m)$$

where dc and dp are respectively the column diameter and the particle diameter of packing and F is the flow-rate.

The optimum value of flow-rate given by this equation is 5.3 mL/min with pure CO_2, but since this value depends directly on the diffusion coefficient Dm of solutes in the mobile phase,[8] the 3 mL/min value will be a good compromise since Dm decreases when modifier is added.

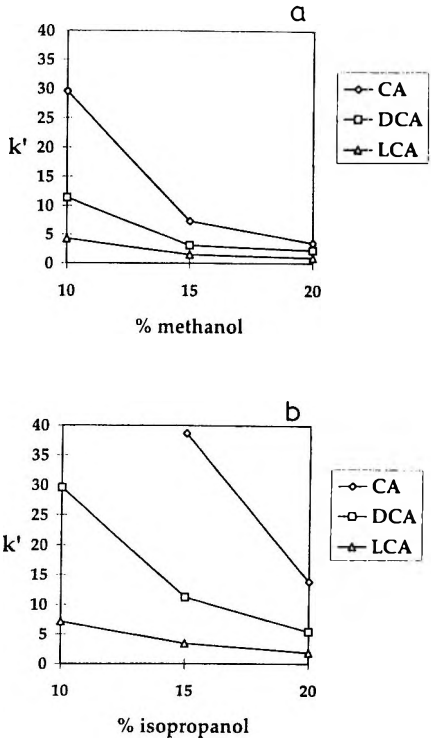

Figure 2. Variation of capacity factors of bile acids with the percentage of modifier in the mobile phase. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 μm; mobile phase, (a) CO_2-methanol, (b) CO_2-isopropanol; flow-rate, 3 mL/min; inlet pressure, 170 bar; temperature, 50°C; detection, ELSD; solutes CA, DCA, LCA.

Retention as a Function of the Modifier

Figure 2 illustrates the variation of the bile acid retention versus the modifier content with two organic modifiers: methanol and isopropanol. The capacity factors decrease with increase in the content of methanol or isopropanol, caused by interactions of the polar modifier with diol groups of stationary phase and enhancement of the solubility of analytes in the

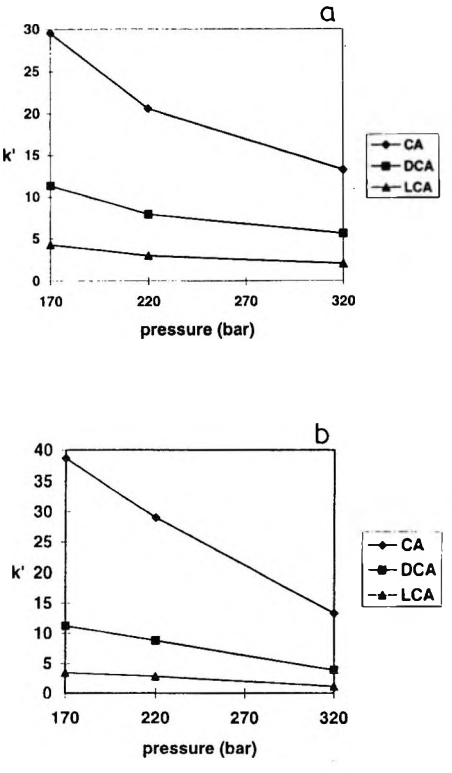

Figure 3. Variation of capacity factors of bile acids with the inlet pressure. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 μm; mobile phase, (a) CO_2-methanol, (b) CO_2-isopropanol; flow-rate, 3 mL/min; temperature, 50°C; detection, ELSD; solutes, CA, DCA, LCA.

mobile phase. In both cases, retention increases in the order monohydroxy (LCA) < dihydroxy (DCA) < trihydroxy (CA). This behaviour is usually observed in a normal phase chromatography.

When we compare both modifier effects, a similar elution strength can be noted for a 10% methanol content and 15% isopropanol content, which illustrates the higher elution strength of methanol and confirms the proposed mechanism .

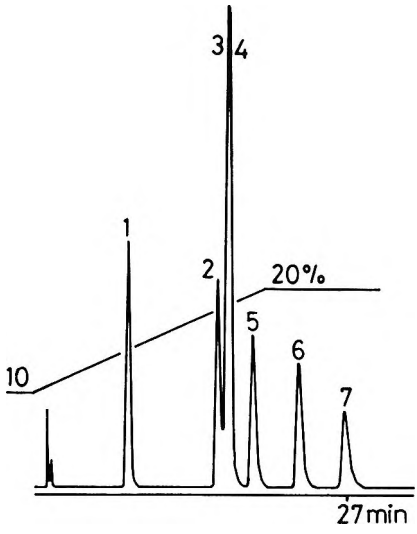

Figure 4. SFC chromatogram of a bile acid mixture. Column, 150 x 4.6 mm I.D. Lichrosorb Diol, 5 μm; mobile phase CO_2 (A) - isopropanol (B), gradient elution from A/B (90:10) to A/B (80:20) in 20 min.; flow-rate: 3mL/min; pressure 170 bar; column temperature : 45°C; solutes : 1=LCA, 2=DCA, 3=CDCA, 4=UDCA, 5=HDCA, 6=HCA, 7=CA.

Table 1

Selectivity as a Function of the Modifier

% Modifier	DCA/LCA	CDCA/DCA	UDCA/CDCA	HDCA/UDCA	HCA/HDCA	CA/HCA
10	2.65	1.09	1.27	1.04	1.67	1.07
15	2.10	1.09	1.24	1.00	1.62	1.07
% Isopropanol						
15	3.30	1.13	1.00	1.34	1.63	1.40
20	2.94	1.00	1.00	1.21	1.52	1.38

Pressure: 170 bar.

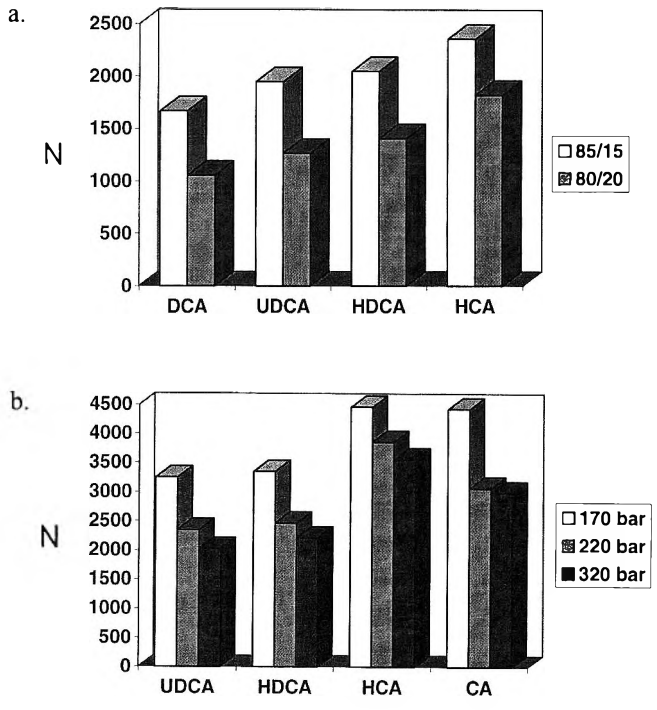

Figure 5. Influence of experimental conditions on column efficiency. Conditions as Fig. 2 except mobile phase and pressure. (a) mobile phase, CO_2- isopropanol (80:20 or 85:15, v/v); (b) mobile phase, CO_2-methanol (90:10), inlet presssure, 170, 220 or 320 bar.

Retention as a Function of Pressure

As shown in Fig. 3, the retention of bile acids decreases, as pressure increases, by enhancing the density and the eluting power of the mobile phase. Comparison of both Fig. 2 and 3 shows that the influence of polar modifier on the retention on SFC packed column is higher than that of the pressure.

As an illustration of the effect of modifier or pressure on retention, Fig. 4 shows the possibility of using a gradient of modifier without baseline drift. This is a major advantage of the use of ELSD over UV detection at low wavelength which requires a high quality grade for such gradient elution.

Table 2

Selectivity as a Function of Pressure

CO_2-Methanol 90:10

Pressure (Bar)	DCA/LCA	CDCA/DCA	UDCA/CDCA	HDCA/UDCA	HCA/HDCA	CA/HCA
170	2.65	1.10	1.27	1.04	1.67	1.07
220	2.63	1.10	1.23	1.05	1.67	1.09
320	2.74	1.08	1.19	1.06	1.59	1.09
CO_2-Isopropanol 85:15						
170	3.30	1.13	1.00	1.34	1.63	1.40
220	3.14	1.12	1.00	1.32	1.61	1.38
320	3.53	1.12	1.00	1.34	1.62	1.41

Selectivity as a Function of the Modifier

With an average efficiency of 2000 theoretical plates and capacity factors from 5 to 30, selectivity values from 1.12 to 1.10, are respectively required for a resolution of one unit. Table 1 gives the selectivities of consecutive solutes obtained using various mobile phases. It appears that the selectivity varies very weakly with the modifier content, but in contrast, it depends on the nature of the modifier.

For example, the selectivity of the couple of dihydroxyl isomers HDCA/UDCA is low (1.00-1.04) with methanol modifier, whatever the modifier content, since high selectivities are obtained (1.21-1.34) with isopropanol. In contrast, the selectivity of the two dihydroxyl isomers UDCA/CDCA is better (1.24-1.27) with methanol, than with isopropanol. The same drastic result is noted for the trihydroxyl isomers CA/HCA couple: selectivity is 1.07 with methanol and about 1.39 with isopropanol.

Resolution and sensitivity can be improved by comparing of efficiency as a function of the modifier content. As the temperature of the column was 50°C, the addition of modifier means the mobile phase is under subcritical conditions; consequently the diffusion coefficients and the efficiency are reduced in

comparison with supercritical conditions. Fig. 5a illustrates the influence of an increase of modifier content at constant pressure: since the viscosity of the mobile phase increases, the efficiency is lowered.

Selectivity as a Function of Pressure

With CO_2-methanol (90:10) at 3 mL/min, a 320 bar pressure permits an analysis time of 15 min which is increased up to 30 min with 170 bar, with no changes in the separation. In fact, the selectivity is not influenced by the pressure variation as shown in Table 2. An increase in pressure at constant modifier content, increases the viscosity of mobile phase and simultaneously decreases the diffusion coefficient of solutes, lowering the efficiency as shown in Fig. 5b.

In conclusion the pressure and modifier content will be used to regulate the analysis time and the nature of organic modifier to enhance the separation parameters.

Influence of the Addition of Water in Organic Modifier

With methanol or isopropanol used as modifier, the complete resolution of the seven bile acids is not obtained. For this reason, the effect of water in polar modifier was also examined. Increasing the amount of water in alcohol results in a slow decrease in retention. However, Table 3 shows that the addition of a small amount of water in methanol as modifier increases only weakly the selectivity of UDCA/CDCA couple, whatever the pressure.

With isopropanol as modifier, the addition of water similarly increases the selectivity of UDCA/CDCA couple but, decreases drastically the selectivity of CA/HCA couple. The CO_2-modifier (85:15) mobile phase with 5% water in isopropanol, allows a separation of nearly all compounds, but the resolution of UDCA/CDCA couple is poor (0.67) and the analysis time is long (34min).

Complete Resolution of Seven Bile Acids

Neither of the two modifiers allows complete separation of the seven bile acids. Results given in Table 1 show the complementarity of CO_2-methanol and CO_2-isopropanol in selectivity between dihydroxyl isomers DCA, UDCA, CDCA and HDCA.

Table 3

Influence of Water Content on the Selectivity

CO_2/Modifier (90:10) 320 Bar
Modifier: Methanol-Water Mixture

% Water in MeOH	DCA/LCA	CDCA/DCA	UDCA/CDCA	HDCA/UDCA	HCA/HDCA	CA/HCA
0	2.74	1.08	1.19	1.06	1.59	1.09
2	2.41	1.07	1.25	1.06	1.55	1.06
5	2.46	1.07	1.26	1.06	1.58	1.06

CO_2/Modifier (90:10) 170 Bar
Modifier: Methanol-Water Mixture

0	2.65	1.10	1.27	1.04	1.67	1.07
2	2.65	1.09	1.39	1.00	1.66	1.00
5	2.67	1.09	1.38	1.00	1.60	1.03

CO_2/Modifier (85:15) 170 Bar
Modifier: Isopropanol-Water Mixture

% Water in Isopropanol						
0	3.30	1.13	1.00	1.34	1.63	1.40
5	3.42	1.11	1.07	1.34	1.68	1.17
8	3.21	1.12	1.10	1.42	1.94	1.00

CO_2/Modifier (80/20) 170 Bar
Modifier: Isopropanol-Water Mixture

0	2.93	1.00	1.00	1.21	1.52	1.38
2	2.57	1.12	1.00	1.20	1.52	1.28
5	2.44	1.08	1.07	1.23	1.53	1.15
8	2.58	1.10	1.09	1.29	1.52	1.07

That is why a mixture of both modifiers was tried to separate these four isomeric solutes. Figure 6 shows the difficult separation of CDCA/DCA and HDCA/UDCA with methanol as modifier (Fig. 6a), the difficult separation of UDCA/CDCA with isopropanol as modifier (Fig. 6b) and the complete separation by mixing both modifiers (Fig. 6c).

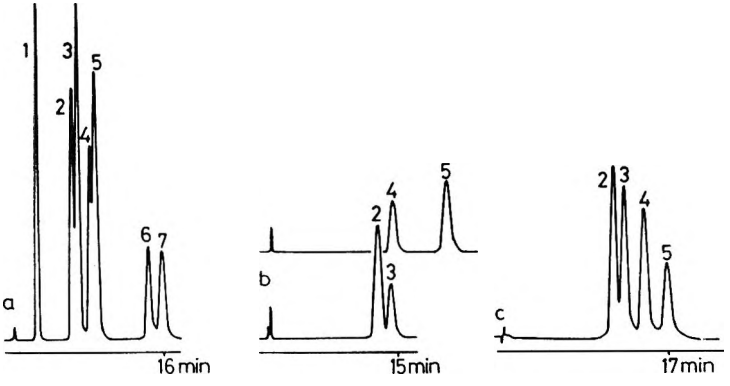

Figure 6. SFC of bile acids. Conditions as in Fig. 2 except (a) CO_2-methanol (90:10, v/v); inlet pressure, 320 bar; (b) CO_2-isopropanol (85:15, v/v); inlet pressure, 170 bar; (c) CO_2-methanol-isopropanol (90:5:5); inlet pressure : 320 bar, solutes : see Fig. 4.

Quantitative Determination of Free Bile Acid in Pharmaceutical Preparation

As determined for liquid phases, the ELSD response in SFC is nonlinear. Using logarithmic coordinates, the response exhibits a linear variation as a function of the concentration C in mg/L. For UDCA as analyte, the response is Log A = 1.70 Log C + 5.36 (r=0.999) with CO_2-methanol (90:10) where A is the peak area. The slope value is similar to that reported for pyrene response.[7]

This value, higher than 1, is interesting for trace component analysis[9] because both detection limit and resolution are increased. With this mobile phase the detection is 800 pmoles for a signal-to-noise ratio S/N 9.6. It becomes 200 pmoles for a similar ratio, using a CO_2-modifier (80:20) with a water content of 5% in isopropanol as modifier.

UDCA has been determined in pharmaceutical preparation. The content of the preparation is dissolved in methanol then filtered and injected into the chromatographic system. This rapid, easy and simple method affords a good recovery compared to the value given by the manufacturer. Moreover, owing to the good sensitivity of this quasi universal detection, Fig. 7 shows the absence in the pharmaceutical preparation of other bile acids (over 0.2 - 0.5 % relatively to UDCA) in particular LCA and DCA which are commonly present in raw material.

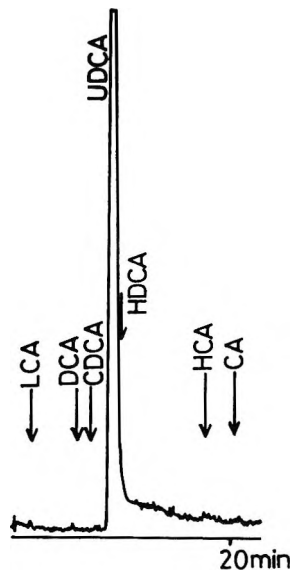

Figure 7. SFC chromatogram of a UDCA in pharmaceutical preparation. Column: Lichrosorb Diol, 150 x 4.6 mm; Mobile phase : CO_2-polar modifier (90:10), polar modifier : methanol-isopropanol (50:50); flow-rate : 5 mL/min; Inlet pressure : 245 bar, outlet pressure : 220 bar; column temperature : 60°C, nebulizing temperature : 50°C. UDCA concentration in methanol (2000 mg/l).

This chromatographic method is more sensitive (about a thousand times) than UV detection and could be applied subsequently to biological samples such as stools.

REFERENCES

1. J. H. Nichols, R. D. Ellefson, J. Hermansen, K. Schifferdecker, M. F. Burritt, J. Liq. Chromatogr., **16(3)**, 681 (1993).

2. A. Roda, C. Cerre, P. Simoni, C. Polimeni, C. Vaccari, A. Pistillo, J. Lipid Res., **33(9)**, 1393 (1992).

3. A. Roda, R. Gatti, V. Cavrini, C. Cerre, P. Simoni, J. Pharm. Biomed. Anal., **11(8)**, 751 (1993).

4. S. Scalia, D. E. Games, J. Pharm. Sci., **82(1)**, 44 (1993).

5. S. Scalia, J. Chromatogr. Biomed. Applications, **431**, 259 (1988).

6. M. Dreux, M. Lafosse, Analusis, **20**, 587 (1992).

7. P. Carraud, D. Thiebaut, M. Caude, R. Rosset, M. Lafosse, M. Dreux, J. Chromatogr., **25**, 395 (1987).

8. F. Verillon, D. Heems, B. Pichon, K. Coleman, J. C. Robert, American Laboratory, June 1992.

9. L. E. Oppenheimer, T. H. Mourey, J. Chromatogr., **323**, 297 (1985).

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J. LIQ. CHROM. & REL. TECHNOL., 19(11), 1819-1830 (1996)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FENBENDAZOLE AND ITS METABOLITES, SULPHOXIDE AND SULPHONE, IN FISH MUSCLE TISSUE

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ABSTRACT

An HPLC method for the determination of fenbendazole (FBZ) and its metabolites, fenbendazole sulphoxide (oxfendazole. FBZ-SO) and fenbendazole sulphone (FBZ-SO_2) in trout and eel tissue is described. The compounds are extracted with ethyl acetate and the extract, after addition of hexane, is concentrated and cleaned up on a silica gel solid-phase extraction column. After elution with 3% acetic acid in methanol the reconstituted eluate is analysed on a Lichrosorb RP8 column, the mobile phase being 0.05 M ammonium phosphate (pH=5): acetonitrile:methanol (55:28:17,v/v). Detection is performed at 297 nm. The average recovery in trout muscle tissue over the concentration range 10-500 µg/kg is 75.7±3.3 % and 77.5±4.1 % for FBZ-SO and FBZ-SO_2, respectively, and for FBZ the recovery is 76.3±5.5 % over the range 15-750 µg/kg.

The average recovery in eel muscle tissue over the concentration range 10-500 μg/kg is 83.5±4.5 % and 87.4±5.2 % for FBZ-SO and FBZ-SO_2 respectively and for FBZ the recovery is 81.4±5.3 % over the range 15-750 μg/kg.

The limits of detection are 3.0, 3.5 and 2.0 μg/kg for FBZ, FBZ-SO and FBZ-SO_2. respectively.

INTRODUCTION

Fenbendazole (FBZ) is an effective broad-spectrum anthelmintic belonging to the benzimidazole class of drugs. It is widely used in mammals to control internal worm parasites. More recently, FBZ is also applied in farmed fish to treat tape-worm infections.[1,2]

Metabolism studies in the goat,[3] in cattle[4] and in the rabbit[5] showed that the major metabolites of FBZ were fenbendazole sulphoxide (oxfendazole. FBZ-SO) and fenbendazole sulphone (FBZ-SO_2). These metabolites were also found in salmon using ^{14}C FBZ.[6] Short et al.[7] studied as well the oxidative metabolism of FBZ in hepatic fractions prepared from liver, among others, in catfish. In that study they could demonstrate the presence of FBZ-SO but not of FBZ-SO_2.

For the assessment of residue levels including these main metabolites in tissues of farmed fish an analytical procedure for the determination of FBZ, FBZ-SO and FBZ-SO_2 should be available. Methods of determination of FBZ, whether or not in combination with FBZ-SO, or with other benzimidazoles in tissue samples,[8,9,10,11,12] or in milk,[13,14,15,16] have been described.

For the determination of FBZ including the main metabolites FBZ-SO and FBZ-SO_2 only a few methods are described. These are in plasma, urine, faeces and tissue homogenates,[17] bovine liver,[18] in plasma[19,20] and in milk.[15,21] The methods for tissue are less or more laborious to be used as starting point for a method suitable for the determination of FBZ and its metabolites in fish muscle tissue.

This paper describes a rapid sample preparation method for the determination of FBZ in fish muscle tissue using solid-phase extraction (SPE) for clean-up and concentration. The method of Steenbaar et al.[22] developed for the determination of mebendazole in eel muscle tissue, was taken as starting point.

EXPERIMENTAL

Reagents and Chemicals

Water was purified via a Milli-Q system (Millipore, Bedford, MA, USA). Methanol, acetonitrile and anhydrous sodium sulphate were from Merck (Darmstadt. Germany). Acetic acid (99-100%), ammonium phosphate, dimethyl sulphoxide, potassium carbonate and silica gel (40 μm. 60 Å) were from J. T. Baker (Phillipsburg, NJ, USA). Ethyl acetate and n-hexane were from Rathburn (Walkerburn, UK). All organic solvents were HPLC grade.

Filter paper circles (S&S 589.1, diameter 90 mm) were from Schleicher and Schüll (Dassel, Germany). Filtration columns, (3 mL) were from J. T. Baker. FBZ was from Riedel-de Haën (Seelse, Germany). FBZ-SO and FBZ-SO_2 were a gift from Syntex (Palo Alto, California, USA).

Stock solutions of FBZ (1.5 mg/mL), FBZ-SO (1 mg/mL) and FBZ-SO_2 (1 mg/mL) were prepared by dissolving 30, 20 and 20 mg, respectively, in 20 mL of dimethyl sulphoxide. A working solution of FBZ (15 μg/mL), FBZ-SO (10 μg/mL) and FBZ-SO_2 (10 μg/mL) was prepared by diluting the stock solutions in methanol.

Standard solutions for HPLC were prepared in the range of 0.0225 - 2.25 μg/mL for FBZ and 0.015 - 1.5 μg/mL for FBZ-SO and FBZ-SO_2 by diluting the working solution in HPLC mobile phase.

A silica SPE column was prepared by weighing 1 g of silica gel in a filtration column with a fritted disk. The column was tapped for 30 s and a fritted disk was placed on the silica bed.

Just before use, the column was pretreated by passing 6 mL of ethyl acetate-hexane (1:4. v/v). After this the column should not allowed to run dry. The SPE elution solvent was 3 % (v/v) acetic acid in methanol.

An 0.05M ammonium phosphate buffer pH 5.0 was prepared by dissolving 5.75 g of ammonium phosphate ($NH_4H_2PO_4$) in 950 mL of water, adjusting the pH to 5.0 with 1 M NaOH and adjusting to 1000 mL with water.

The mobile phase for HPLC was methanol-acetonitrile-0.05 M ammonium phosphate buffer pH 5.0 (17:28:55, v:v) and was degassed before use.

Apparatus and Chromatographic Conditions

The instruments used were a Moulinette homogenizer (Moulinex, Gouda, The Netherlands), a KS 500 mechanical shaker (IKA-Labortechnik. Janke and Kunkel, Staufen, Germany), a Vibrofix VF 1 vortex mixer (IKA Labortechnik), a Centra-8R centrifuge (IEC, Needham, MA, USA), a 5414c Eppendorf centrifuge (Hamburg, Germany), a Reacti-Therm III heating module and a Reacti-Vap III evaporator (Pierce, Rockford, IL, USA), a SPE-21 column processor (J.T. Baker) and an ultrasonic bath (Branson, Soest, The Netherlands).

The HPLC system consisted of a 2150 HPLC pump (Pharmacia-LKB, Uppsala, Sweden), a 2153 autosampler (Pharmacia-LKB) with a 50-µL sample loop, and a 783A HPLC monitor with a 10-µL HPLC flow cell (Applied Biosystems, Foster City, CA, USA) operated at 297 nm.

A ChromSep cartridge holder system contained a stainless-steel guard column (10 mm x 2.1 mm I.D.) packed with pellicular 40-µm reverse phase particles and two coupled analytical glass columns (2 x 100 mm x 3.0 mm I.D.) packed with 5-µm Lichrosorb RP 8 (Chrompack, Bergen op Zoom, The Netherlands). The system was operated at ambient temperature. Peak areas were quantitated with an SP 4270 integrator (Spectra-Physics, San Jose, CA. USA).

Samples

For recovery studies blank trout and eel muscle tissue were spiked at levels of 15-750 µg/kg for FBZ, and 10-500 µg/kg for FBZ-SO and FBZ-SO_2 at least 15 min before extraction. When samples were not directly analysed they were stored at -20 °C.

Sample Preparation

Extraction. Ground trout or eel muscle tissue was accurately weighed (*ca.* 5 g) into a 50 mL disposable plastic centrifuge tube and 0.5 mL of 4 M potassium carbonate solution was added. The mixture was vortexed for 10 s and 10 mL of ethyl acetate was added. The tube was shaken again on a vortex mixer for 10 s. and placed on a mechanical shaker for 10 min (500 rpm). The suspension was centrifuged for 6 min at 3400 g. The supernatant was decanted. The extraction procedure was repeated with another 10 mL of ethyl acetate.

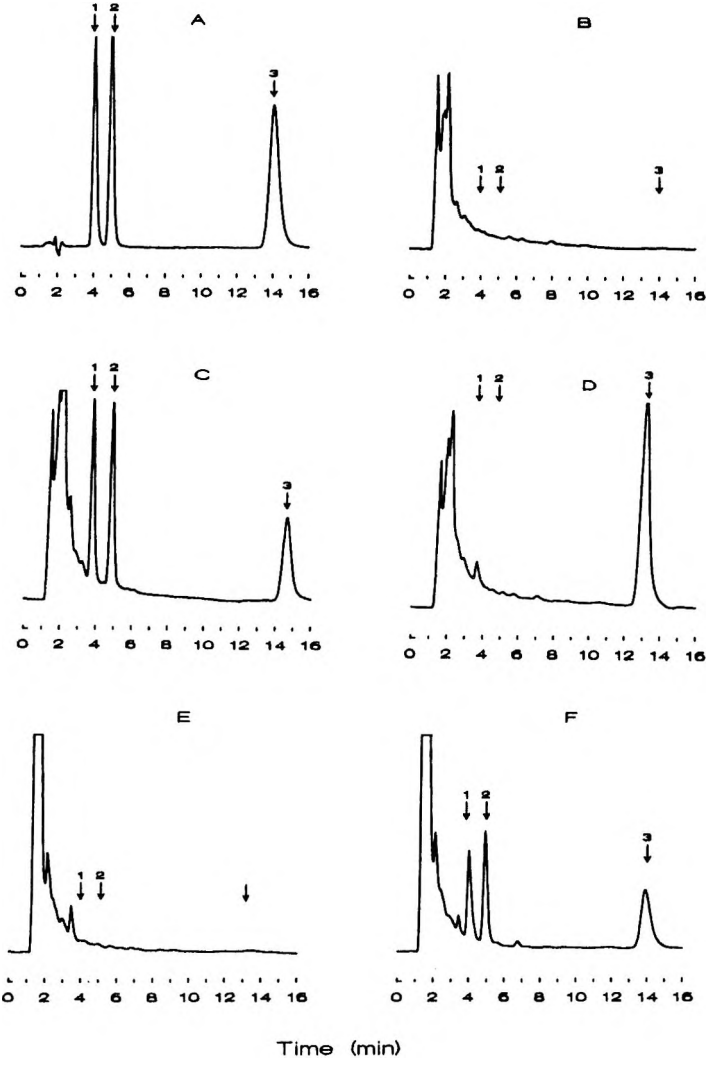

Figure 1: Chromatograms of (A) a standard solution of 500 µg/L of FBZ-SO (1), 500 µg/L FBZ of FBZ-SO_2 (2) and 750 µg/L of FBZ; (3) (B) a blank trout muscle tissue; (C) a trout muscle tissue sample spiked with 100 µg/kg of FBZ-SO,(1) 100 µg/kg of FBZ-SO_2 (2) and 150 µg/kg of FBZ (3); (D) a real trout muscle tissue sample containing 7 µg/kg of FBZ-SO (1) and 163 µg/kg of FBZ (3) ; (E) a blank eel muscle tissue sample; (F) an eel muscle tissue sample spiked with 50 µg/kg of FBZ-SO (1), 50 µg/kg FBZ-SO_2 (2) and 75 µg/kg of FBZ (3).

Clean up and concentration. 80 mL of hexane were added to the combined extracts (the ethyl acetate-to-hexane ratio should be *ca* 1:4 v/v). After the addition of 2 g of anydrous sodium sulphate, the solution was shaken and allowed to stand until it had become transparent. The solution was filtered over an S & S 589.1 filter paper circle, and the filtrate was passed through the pretreated silica gel SPE column via a 75-mL reservoir. The column was dried in a stream of nitrogen for 10 min. Then, FBZ, FBZ-SO and FBZ-SO_2 were eluted with 3 mL of 3% acetic acid in methanol. The eluate was evaporated to dryness in a stream of nitrogen at 37 °C.

The residue was dissolved in 550 μL of 0.05 M ammonium phosphate. Then, 280 μL of acetonitrile was added, followed by 170 μL of methanol. This solution was placed in an ultrasonic bath for 5 min, and centrifuged for at least 8 min at 15800 g.

Chromatography

Aliquots (50 μL) of the sample and standard solutions were injected. Samples were eluted isocratically at a flow rate of 0.6 mL/min.

RESULTS AND DISCUSSION

Chromatography

Chromatography of FBZ is usually carried out on reversed-phase columns. If metabolites are taken into account as well there is a problem with respect to retention. There is a significant difference in polarity of FBZ and FBZ-SO, resulting in a strong difference in retention times in most systems. If FBZ-SO has any retention then the retention time of FBZ is usually very high. To overcome this problem gradient elution was used[17,18,21] or different mobile phases.[9,13] As these systems are less or more complicated, attention was paid to the development of a suitable eluent including ion-pair systems in combination with different types of reversed phases. The optimal system proved to be a 20 cm Lichrosorb RP8 column with 0.05 M ammonium phosphate (pH=5): acetonitrile : methanol = 55:28:17 (v/v) at a flow rate of 0.6 mL/min, the retention times being *ca.* 4 min, 5 min, and 15 min for FBZ-SO, FBZ-SO_2 and FBZ, respectively.

Detection was performed at 297 nm. A typical chromatogram of a standard solution of FBZ, FBZ-SO and FBZ-SO_2 using the developed system is shown in Figure 1A.

Sample Preparation

In the method of Steenbaar *et al.*[22] extraction of mebendazole was performed with ethyl acetate after the addition of sodium sulphate and a potassium carbonate solution to the ground tissue. However, for FBZ low extraction recoveries were obtained, which were caused by the addition of sodium sulphate to the tissue. Therefore, sodium sulphate was added after extraction to remove the water originally present in the fish muscle tissue. This was necessary as the presence of water in the ethyl acetate extract has a negative effect on the retention of the analytes on the silica SPE column.

Next, the ratio ethyl acetate-to-hexane 1:2.5 (v/v) was changed to 1:4 (v/v) for complete retention of all three compounds on the silica SPE column. For the same reason the amount of silica sorbent in the SPE column has to be enlarged from 0.5 g to 1 g.

Further. the dissolution of the residue, prior to HPLC, proved to be very critical for the recovery of FBZ. The residue had to be dissolved into the buffer first, followed by addition of acetonitrile and methanol, instead of dissolution in mobile phase. Otherwise low recoveries were found. However, the obtained solution was not clear.

Attempts were made to make the solution transparent by extraction with n-hexane, iso-octane or petroleum ether. But there were losses of FBZ into the extractant being approximately 5-8%. Finally, centrifugation proved to be successfully.

Spiking Studies and Real Samples

Recovery experiments (6 replicates) were carried out on trout muscle tissue spiked at 10. 50 and 200 μg/kg for FBZ-SO and FBZ-SO_2, and 15, 75 and 300 μg/kg for FBZ. In addition some recovery experiments were carried out in triplicate. These results are presented in Table 1. The calibration curve is linear throughout the range 15-750 μg/kg for FBZ (r=0.998) and 10-500 μg/kg for FBZ-SO and FBZ-SO_2 (r=0.999 and 0.999, respectively). The same experiments were carried out on eel muscle tissue. These results are presented in Table 2.

The calibration curve is linear throughout the range 15-750 μg/kg (r=0.999) for FBZ and 10-500 μg/kg for FBZ-SO and FBZ-SO_2 (r=0.999 and 0.999, respectively). A good recovery at all the levels investigated and a low standard deviation (SD) for repeatability are attained for all three compounds.

Table 1

The Recovery of Analytes from Trout Muscle Samples Spiked at Different Levels

	FBZ		FBZ-SO		$FBZ-SO_2$	
N	Level Tg/kg	Recovery[a] (%)	Level Tg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)
6	15	77.0±2.7	10	74.5±2.8	10	74.1±3.4
3	30	81.0±1.0	20	79.0±2.8	20	75.2±2.8
6	75	69.5±2.3	50	71.8±1.8	50	74.5±1.9
6	150	79.5±2.9	100	76.8±2.7	100	80.9±2.6
3	300	80.9±1.7	200	79.2±0.2	200	83.3±0.5
3	750	72.6±2.1	500	77.1±0.4	500	80.3±0.7

N = Number of replicates
[a] Mean ± S.D.

Table 2

The Recovery of Analytes from Eel Muscle Samples Spiked at Different Levels

	FBZ		FBZ-SO		$FBZ-SO_2$	
N	Level µg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)	Level µg/kg	Recovery[a] (%)
6	15	79.1±4.3	10	86.3±6.7	10	89.6±6.6
2	30	75.6±0.3	20	75.3±2.3	20	75.7±2.3
6	75	79.8±3.1	50	82.7±1.7	50	86.8±2.4
6	150	93.4±2.0	100	80.9±4.8	100	84.1±0.5
3	300	81.7±2.2	200	85.3±2.1	200	90.7±1.6
3	750	83.9±0.5	500	85.5±1.8	500	90.8±1.0

N = Number of replicates
[a] Mean ± S.D.

Table 3

The Recovered Analyte Content of Spiked (75 μg/kg of FBZ and 50 μg/kg of FBZ-SO and 50 μg/kg of FBZ-SO_2) Trout Muscle Tissue Test Portions Analyzed on Different Days

		Analyte Content		
Day	**N**	**FBZ[a] (μg/kg)**	**FBZ-SO[a] (μg/kg)**	**FBZ-SO_2[a] (μg/kg)**
1	6	54.5±0.6	36.0±0.6	38.8±0.6
2	6	52.1±1.7	35.8±0.9	27.2±1.0
3	4[b]	57.9±2.5	40.2±4.6	41.6±5.0
4	6	53.8±3.3	40.2±0.9	41.7±1.9
5	6	58.7±2.2	39.4±0.6	40.5±0.8
6	5[c]	54.3±1.6	37.8±0.8	38.3±0.8
Overall mean	33	55.1±3.1	39.3±1.9	37.9±1.9

[a] Mean±S.D.
[b] Two samples lost
[c] One sample lost

The day-to-day variation was studied as well. Blank trout muscle tissue was spiked at 75 μg/kg of FBZ and 50 μg/kg of FBZ-SO and FBZ-SO_2. The spiked test portions were analysed in 6 replicates on 6 different days, following the procedure outlined above. The results are shown in Table 3. A reasonable SD of the mean for all compounds was attained, the relative SD being 5.6 %. 4.8 % and 5 % for FBZ, FBZ-SO and FBZ-SO_2, respectively.

Typical chromatograms of blank, and spiked trout and eel muscle tissue are shown in Figure 1B, 1C, 1E, 1F.

The absolute limits of detection of FBZ, FBZ-SO and FBZ-SO_2 were 0.5, 0.6 and 0.2 ng, respectively,on-column, at a signal-to-noise ratio of 3.0. As no interferences of endogeneous compounds from tissue at the retention time were found, the limits of detection were 3.0, 3.5, and 2.0 μg/kg for FBZ, FBZ-SO and FBZ-SO_2, respectively. in muscle tissue. Levels of 6.0, 7.0 and 4.0 μg/kg for FBZ. FBZ-SO and FBZ-SO_2. could easily be quantitated in these tissues.

As an illustration of the applicability of the described method muscle tissue of FBZ treated trout was analysed. To obtain real samples FBZ was administered orally. by intubation of the stomach, to two trouts *(Oncorhynchus mykiss)* with a mean body weight of 110 g. The dose was 6 mg/kg body weight, which is equivalent to 0.24 mL of Panacur suspension 2.5% w/v (Hoechst. Amsterdam, The Netherlands) per kg of fish weight. The contents of FBZ and FBZ-SO formed in tissue, were 139 μg/kg and 8 μg/kg, respectively after 24 h of the administration. FBZ-SO_2 was not detected. A chromatogram of a real sample is shown in Figure 1D.

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REFERENCES

1. R. Nordomo, "Approach to Salmon Farming in Norway",in **Aquaculture for Veterinarians,** L. Brown. ed., Pergamon Press, Oxford. 1993. pp.179-191.

2. O. Torrisen , I. Opstad, O. M. Rodseth. "The Veterinary Approach to Cod," in **Aquaculture for Veterinarians**, L. Brown. ed.. Pergamon Press, Oxford, 1993, pp.345-356.

3. C. R. Short, S. A. Barker, L. C. Hsieh, S-P. Ou, L. E. Davis, G. Koritz, C. A. Neff-Davis, R. F. Bevill, I. J. Munsiff, Am. J. Vet. Res., **48**, 811-815 (1987).

4. C. R. Short, S. A. Barker, L. C. Hsieh, S-P. Ou, T. McDowell, L. E. Davis, C. A. Neff-Davis, G. Koritz, R. F. Bevill, I. J. Munsiff, Am. J. Vet. Res., **48**, 958-961 (1987).

5. C. R. Short, S. A. Barker, L. C. Hsieh, S-P. Ou, T. McDowell, Res. Vet. Sci., **44**, 215-219 (1988).

6. I. Nafstad, K. Ingebrigtsen, K. Langseth, H. Hektoen, I. L. Gross, B. Bergsjo, Acta Vet. Scand. Suppl., **87**, 302-304 (1991).

7. C. R. Short, W. Flory, L. C. Hsieh, S. A. Barker, J. Vet. Pharmacol. Ther., **11**, 50-55 (1988).

8. A. M. Marti, A. E. Mooser, H. Koch, J. Chromatogr., **498**, 145-157 (1990).

9. L. W. Levan, C. J. Barnes, J. Assoc. Off. Anal. Chem., **74**, 487-493 (1991).

10. R. T. Wilson, J. M. Groneck, A. C. Henry, L. D. Rowe, J. Assoc. Off. Anal. Chem., **74**, 56-67 (1991).

11. W. J. Blanchflower, A. Cannavan, G. Kennedy, in **Residues of Veterinary Drugs in Food**, N. Haagsma, A. Ruiter, P. B. Czedik-Eysenberg, eds., Proceedings of the EuroResidue II Conference, Veldhoven, The Netherlands, 1993, pp.191-195.

12. W. J. Blanchflower, A. Cannavan, D. G. Kennedy, Analyst, **119**, 1325-1328 (1994).

13. A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short, S. A. Barker, J. Assoc. Anal. Chem., **72**, 739-741 (1989).

14. S. S-C. Tai, N. Cargile, C. J. Barnes, J. Assoc. Anal. Chem., **73**, 368-373 (1990).

15. D. J. Fletouris, N. A. Botsoglou, I. E. Psomas, A. I. Mantis, Analyst, **119**, 2801-2804 (1994).

16. D. Nakos, N. A. Botsoglou, I. E. Psomas, J. Liq. Chromatogr., **17**, 4145-4155 (1994).

17. S. A. Barker, L. C. Hsieh, C. R. Short, Anal. Biochem., **155**, 112-118 (1986).

18. S. A. Barker, T. McDwell, B. Charkhian, L. C. Hsieh, C. R. Short, J. Assoc. Anal. Chem., **73**, 22-25 (1990).

19. K. H. Lehr, P. Damm, J. Chromatogr., **382**, 355-360 (1986).

20. M. S. Bull, G. R. E. Shume, J.Pharm. Biomed. Anal., **5**, 501-508 (1987).

21. P. K. Sanyal, Vet. Q., **15**, 157-159 (1993).

22. J. G. Steenbaar, C. A. J. Hajee, N. Haagsma, J. Chromatogr., **615**, 186-190 (1993).

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THE USE OF SOLID PHASE EXTRACTION CARTRIDGES AS A PRE-FRACTIONATION STEP IN THE QUANTITATION OF INTERMOLECULAR COLLAGEN CROSSLINKS AND ADVANCED GLYCATION END-PRODUCTS

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ABSTRACT

A range of solid phase extraction (SPE) sorbents was investigated to determine their suitability as a pre-fractionation step during the quantitation of collagen crosslinks and advanced glycation end products by reverse phase high performance liquid chromatography (RP HPLC).

Propylsulphonic acid (PRS) SPE cartridges were shown to be the most suitable sorbent type by testing for resolution of specific amino acids using both inorganic salt buffers and volatile organic solvents.

The loading capacity of the PRS-SPE cartridges was determined and the efficacy of the cartridges evaluated by comparison with previously published methods using fibrous cellulose and SP Sepharose.

PRS-SPE cartridges provided a superior, rapid, cost effective alternative to either of these pre-fractionation steps giving a higher recovery of both the mature collagen crosslink, pyridinoline, and the advanced glycation end-product crosslink, pentosidine.

INTRODUCTION

Collagen is stabilised by intermolecular crosslinks formed following the enzymic oxidation of specific lysines in the collagen molecule by lysyl oxidase. The initial products are intermediate divalent crosslinks that, with time, are spontaneously converted into trivalent mature crosslinks. {For a review see Bailey.}[1] Quantitation of the intermediate collagen crosslinks dehydro-hydroxylysinonorleucine (Δ-HLNL), hydroxylysinoketo-norleucine (HLKNL) and dehydro-histidinohydroxymerodesmosine (Δ-HHMD) requires their reduction with sodium borohydride to stabilise them prior to± acid hydrolysis. The trivalent, mature collagen crosslinks hydroxylysyl pyridinoline (Hyl-Pyr), lysyl pyridinoline (Lys-Pyr) and histidinohydroxylysinonorleucine (HHL) are all stable to acid hydrolysis without prior reduction.

It is possible for all these crosslinks to be quantified from an acid hydrolysate of reduced, collagenous tissue by ion-exchange chromatography using either ninhydrin or another post-column detection system. Alternatively pyridinoline, being naturally fluorescent, is commonly quantified by exploiting this characteristic using a reverse phase high performance liquid chromatography (RP HPLC) procedure.[2]

Collagen has a long biological half-life and adventitiously accretes glucose by non-enzymic processes. The initial product of this reaction can in turn be oxidised to form several compounds referred to as advanced glycation end-products (AGE's), some of which act as intermolecular crosslinks. {For a review see Paul and Bailey}.[3]

One such crosslinking component has been characterised and named pentosidine, based on its proposed derivation from ribose. Like pyridinoline, pentosidine is naturally fluorescent and can also be quantified by RP HPLC using fluorescence detection.[4]

Due to the paucity of all these crosslinking components in most tissue hydrolysates, it is frequently necessary to undertake a pre-fractionation step to enhance their relative concentrations over the other constituent amino acids. In 1988, Black et al[5] reported a pre-fractionation step using fibrous cellulose (CF1) to concentrate pyridinoline.

This procedure was subsequently modified and shown by Sims and Bailey, in 1992[6], to also be suitable for concentrating both the intermediate and mature collagen crosslinks. However, in our experience CF1 pre-fractionation of hydrolysates for pentosidine quantitation is not reliable since pentosidine is desorbed to a variable degree by the organic eluant.

This variability can be controlled, but not eliminated, by keeping both loading volume and organic wash volume as small as practically possible. In practice this means loading samples in less than 30% of the column volume and limiting the organic washes to no more than 5 column volumes. In this fashion it is possible to keep the losses of pentosidine to considerably less than the 88% reported by Takahashi et al,[7] but is none the less still unacceptably high.

Sell and Monnier[8] reported using sulphopropyl (SP) Sephadex cation exchange columns to purify their fluorescent glycation components, whilst Dyer et al.[9] employed a C_{18} Sep pack cartridge as a pre-fractionation step to concentrate MFP-1, shown to be pentosidine in that same paper.

Takahashi et al.[7] reported better than 70% recovery of pentosidine and pyridinoline standards using the same SP Sephadex cation exchange sorbent compared to better than 70% recovery of pyridinoline but only 12% recovery of pentosidine from CF1

In this paper, we report the results of a detailed survey of normal and reversed phase, solid phase extraction (SPE) cartridges, the selection and testing of propylsulphonic acid (PRS) SPE cartridges and the development of a rapid, simple method for the recovery of both mature collagen crosslinks and AGE standards in good yield using volatile organic buffers.

Finally, we report the results of a comparison of PRS solid phase extraction with CF1 pre-fractionation of twelve whole muscle hydrolysates preceding collagen crosslink quantitation by both amino acid analysis and RP HPLC. We had hypothesised, that variation in muscle growth rate (as indicated by their growth coefficients), might be reflected in variation in the intra-muscular collagen crosslinking and as muscle has such a low collagen content, it provided a rigorous test material for comparison between the established methodologies and the proposed new method.

Table 1

Selected Muscles and their Reported Growth Coefficients

Muscle Name	Growth Coefficient	Relative Growth Group
Gastrocnemius	0.84*	Early
Extensor carpi radialis	0.85	Early
Flexor hallucis longus	0.86	Early
Semitendinosus	0.91*	Average
Psoas major	0.92*	Average
Supraspinatus	0.94	Average
Triceps brachii	0.99	Average
Pectoralis profundus	1.00	Average
Latissimus dorsi	1.05	Late
Semispinalis capitis	1.07*	Late
Serratus ventralis thoracis	1.13	Late
Splenius	1.18	Late

* Averaged value for Multiphasic growth pattern.
Data from Butterfield & Berg.[10]

MATERIALS AND METHODS

Reagent.

Unless otherwise stated, all reagents were Analar grade purchased from Fisons Chemicals (Leicestershire, England). HPLC solvents were purchased from Rathburn Fine Chemicals (Peebleshire, Scotland). Amino acid standards were purchased from Pierce Warriner (Cheshire, England) and crosslink standards were isolated and purified in our own laboratory.

Meat Sample.

A Charolais x Freisian-Holstein steer, 25 months old, was purchased locally and slaughtered under humane, hygienic conditions in the Bristol University abattoir. The carcass was left to hang at ambient temperature (>10°C) overnight, before removal to a chiller for a further 24 hours.

Twelve muscles were selected (Table 1) which, according to their reported growth coefficients,[10] could be categorised as having early, average or late relative growth development and were removed from one side of the carcass.

Samples were processed as described by Avery and Bailey,[11] in brief all the epimysium and associated fat was removed from each of the muscles which were then cut into manageable cubes and separately comminuted in a Moulinette S (Moulinex, France) food processor. 1g aliquots of each muscle were suspended in phosphate buffered saline (PBS) at pH 7 and the intermediate crosslinks stabilised by reduction with nascent hydrogen released from sodium borohydride over a 2 hour period at room temperature.[12]

After pH adjustment to stop the reaction, the samples were washed with distilled water (to remove salt) filtered, freeze-dried, weighed and powdered in the food processor, prior to weighed aliquots (100mg) being hydrolysed in 15mL of constant boiling hydrochloric acid (HCl) at 112°C for 24 hours in hydrolysis tubes sealed after gassing with nitrogen.

SPE Cartridge Type Selection.

100mg, Bond Elut SPE cartridges (Analytichem International Inc. California, USA) of twelve different sorbent types (Table 2), were washed, pre-conditioned and equilibrated in a solvent most suited for analyte binding, according to the information supplied by the manufacturer.

A standard solution of hydroxyproline and lysine (10 mg/mL) in 0.01M HCl, was prepared and 10µl loaded on to each cartridge type. These amino acids were selected to represent the range of pKa characteristics of the majority of amino acids, including the collagen crosslinks, present in a collagenous tissue hydrolysate. Also, hydroxyproline produces a different coloured product when reacted with ninhydrin, making resolution of the two more readily detectable. The loading volume was kept small to minimise cartridge re-conditioning by the 0.01M HCl.

10 bed volumes (1mL) of the equilibration solvent were passed through each cartridge to determine how strongly bound the amino acids were, followed by 10 bed volumes of the suggested elution solvent for each sorbent type. After each bed volume, a 10µl drop of the eluant was dried onto filter paper and sprayed with a 0.2% solution of ninhydrin in acetone and heated at 100°C for 2 minutes to detect the colour of the eluted amino acids.

Table 2

Relative Retention of the Amino Acid Test Mixture (Hydroxyproline & Lysine) by SPE Sorbents

	Elution Position in Equilibrium		Or Eluting Solvent	
Matrix Type	**First 50%**	**Final 50%**	**First 50%**	**Final 50%**
Non-polar				
Octadecyl	+++	++		
Octyl	++++	+		
Ethyl	+++++			
Cyclohexyl	+++++			
Phenyl	+++++			
Polar				
Cyanopropyl	+++++			
Diol	+++++			
Aminopropyl	+++++			
Cation Exchange				
SCX		++++	+	
CBA	++	+++		
Anion Exchange				
SAX	+++++			

Cationic SPE Cartridge Selection

Three types of cation exchange SPE cartridges were obtained from three different manufacturers. (i) CBA cartridges possess a weakly ionic, carboxymethyl group, (ii) PRS cartridges possess a sulphonylpropyl group and are intermediary in ionic strength between CBA and (iii) SCX cartridges which possess a benzenesulphonylpropyl group and are strongly ionic. A range of inorganic buffers of different pH's was used to resolve the same two standard amino acids. The recovery of the hydroxyproline standard from each manufacturers sorbent type was precisely quantified by means of a ChemLab Autoanalyser (Chemlab Instruments Ltd, Essex, England) utilising the method of Bannister and Burns.[13]

The cartridges were prepared as before according to the manufacturers instructions then equilibrated in 0.1M citrate buffer pH 2.1. The sample was loaded in 10µl of 0.01M HCl and eluted with a succession of 1mL washes of

0.1M citrate buffered at pHs 2.1, 3.1, 3.5, 4.7, 6.5 and 8.5 and finally with 4N ammonium hydroxide to displace any residual material. All washes were collected and the recovery of hydroxyproline determined as described above. In addition, 10µl aliquots were spotted on to filter paper and sprayed with ninhydrin, as before, to determine the elution position of lysine.

SP Sepharose Pre-fractionation v. PRS Solid Phase Extraction Cartridges

PRS solid phase extraction cartridges were used to test a standard mixture of the fluorescent, mature collagen crosslink pyridinoline and hydroxyproline. This mixture, also in 0.01M HCl, was loaded on to both 500mg and 1g PRS cartridges (International Sorbent Technology, Glamorgan, Wales) packed in 3mL syringe bodies, to prohibit elution variation due to changes in cartridge dimension, and eluted as before with the citrate buffers. An equal amount of the same standard mixture was loaded on to 4mL and 8mL columns of SP Sepharose (Pharmacia, Buckinghamshire, England) prepared in the laboratory. These Sepharose columns were eluted with 0.15M and 1M HCl, according to the method of Takahashi et al.[7]

All eluants were collected, vacuum concentrated and the recoveries of all components calculated as a percentage of the initial loading. Pyridinoline was quantified by RP HPLC using a Hypercarb S, (Shandon HPLC, Cheshire, England) graphitic carbon column, 100 x 4.6mm, with a gradient from 0-12% tetrahydrofuran in water, each containing 0.5% trifluoroacetic acid and a flow rate of 1mL / minute. The pyridinoline standard was detected by means of its natural fluorescence using an LS5 luminescence spectrophotometer (Perkin-Elmer, Buckinghamshire, England.), with excitation set at 295 nm and emission at 405 nm. Bailey et al.[14]

PRS-SPE Cartridge Preparation and Eluting Buffer Modifications.

Modifications were made to both the cartridge manufacturers preparation procedure and the elution buffers. The initial cartridge "wetting" with methanol, was modified by incorporating 1% triethylamine in the methanol and increasing the wash volume to 10 cartridge volumes. The methanol was displaced with 3 volumes of water containing 10% methanol followed by 3 volumes of 0.1M HCl and finally equilibrated with 3 volumes of loading buffer also containing 10% methanol. The inclusion of methanol was intended to prohibit hydrophobic interactions with the propyl chain of the propylsulphonic acid functional group. A selection of organic volatile buffers (Table 5) was prepared according to published data,[15] covering the same pH range as the citrate buffers and each containing 10% methanol.

PRS Cartridge Loading Capacity

To determine the loading capacity of both the 500mg and 1g PRS cartridges, a known dry weight of bovine serum albumen (BSA) was hydrolysed, then freeze-dried and rehydrated in the volatile loading buffer. A constant volume but variable weight of BSA was added to a constant amount of both standard fluorescent crosslinks, pyridinoline and pentosidine, plus hydroxyproline.

A range from 1 to 15% of the cartridge sorbent dry weight of BSA was loaded on both PRS cartridge sizes and the components eluted using the new volatile buffers. The cartridge eluant at each pH was analysed for hydroxyproline and fluorescent crosslink recovery after vacuum concentration as described above.

CF1 Pre-fractionation v. PRS Solid Phase Extraction Cartridges

After drying and re-hydrating in the respective equilibration solvents, aliquots from the twelve reduced, acid hydrolysed, whole bovine muscles, equal to 30mg, were loaded on to 3 mL CF1 columns, prepared in the laboratory, and 1g PRS-SPE cartridges. The aqueous eluant from the CF1 and the combined pH 3.5 and 4.7 eluants from the SPE cartridges were dried down separately and analysed, according to the method of Sims and Bailey,[6] using an Alpha Plus amino acid analyser (Pharmacia, Buckinghamshire, England). Aliquots of the same eluants from duplicate pre-fractionations were analysed by RP HPLC as previously described.[14]

RESULTS

SPE Cartridge Type Selection

Table 2 shows the relative retention of hydroxyproline and lysine by the 12 different sorbent types from Analytichem International Inc. No resolution of hydroxyproline from lysine by any of the matrices was achieved, as judged by colour development with ninhydrin, all spots appearing blue/grey. However, on the basis of the retention of both the test amino acids until late in the equilibration washes, it was judged worthwhile to undertake a further investigation of the available range of cation exchange SPE cartridges.

Table 3

% Recovery of Hydroxyproline from Cation SPE Sorbents

	Manufacturer 1			Manufacturer 2		Manufacturer 3	
Eluant	**CBA**	**PRS**	**SCX**	**CBA**	**SCX**	**CBA**	**SCX**
Load (pH2.1)	102*	102	0	104*	0	98*	0
pH3.1	0	4.4	0	0	0	0	0
pH3.5	0	3.7	0	0	0	0	0
pH4.7	0	0*	0	0	0	0	0
pH6.5	0	0*	0	0	0	0	0
pH8.5	0	0	0	0	0	0	0
4N	0	0	52.1	0	102.8	0	38.9

* Indicates the elution position of the lysine, i.e. during loading or elution, as judged by ninhydrin spraying.

Cationic SPE Cartridge Selection

Table 3 illustrates the binding strength of the amino acid test mixture to each cationic cartridge type and shows the percent recovery of hydroxyproline from the three different types of SPE cation sorbent.

These results demonstrate that the strong cation exchange (SCX) cartridges bound the standard amino acids too tightly, requiring high salt concentrations to desorb them, which, would result in subsequent problems for amino acid analysis. Conversely, the CBA cartridges failed to bind either standard amino acid effectively. The apparent resolution of lysine and hydroxyproline by the PRS solid phase extraction cartridges suggested that further investigation of these cartridges was worthwhile, however, the elution of very small amounts of hydroxyproline from the same cartridges at higher pHs ("ghosting"), suggested some form of non-specific binding was occurring.

Table 4

% Hydroxyproline and Pyridinoline Recovery from PRS Cartridges v. that from SP Sepharose Columns

	500 mg PRS		1g PRS		4 mL Sepharose		8mL Sepharose	
Eluant	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**	**Hyp**	**X-Lnk**
pH2.1	36	0	0	0				
pH3.1	59	0	0	0				
pH3.5	0	0	92	0				
pH4.7	0	86	0	104				
0.15M					60	0	59	0
1.0M					11	78	16	69

(Values are the average of triplicate loadings.)

SP Sepharose Pre-fractionation v. PRS Solid Phase Extraction Cartridges

Table 4 shows the hydroxyproline and fluorescent crosslink recovery from the PRS cartridges using citrate buffers and from the SP Sepharose columns using dilute HCl. Both the hydroxyproline and pyridinoline recovery from the PRS cartridges, is greater than that from the SP Sepharose columns and the resolution between these standards is more pronounced. In addition, not all the standard mixture seems to have desorbed from the SP Sepharose and continued elution with HCl failed to desorb them. The volume of eluant involved for PRS desorption is significantly smaller than that for SP Sepharose, resulting in considerably shorter run times and faster subsequent processing by freeze-drying or vacuum concentrating.

The recoveries of both pyridinoline and hydroxyproline, from the Sepharose columns, are comparable to each other and similar to those reported by Takahashi et al.[7] for pentosidine and pyridinoline.

During subsequent analysis of these samples several disadvantages of the SPE procedure became apparent: (i) The manufacturers guidelines for cartridge preparation were inadequate, as some components were eluting from the cartridges, especially at high pH, and binding to the HPLC column causing severe retention time variation. (ii) The citrate residues after vacuum concentration were sufficiently large to cause problems for any subsequent amino acid analysis. (iii) The fact that hydroxyproline appeared to "ghost"

Table 5

Volatile Buffer Formulations for PRS Solid Phase Extraction Cartridges

Type / pH of buffer	Components
Washing / "wetting	1% Triethylamine in Methanol
Conditioning I	10% methanol in water
Conditioning II	10% methanol in 0.1N Hcl
Loading / Equilibration (pH 1.9)	87mL glacial acetic acid, 25mL 90% formic acid, 100mL methanol to 1litre
Eluting buffers	
pH 3.1	5mL pyridine, 100mL glacial acetic acid, 100mL methanol to 1L
pH 3.5	5mL pyridine, 50ml glacial acetic acid, 100mL methanol to 1L
pH 4.7	25mL pyridine, 25mL glacial acetic acid, 100mL methanol to 1L
pH 6.5	100 mL pyridine, 4mL glacial acetic acid, 100mL methanol to 1L

(Data, with modification, from Dawson et al.[15]

from both silicon and Sepharose matrices of the propylsulphonic acid functional group suggested that hydrophobic interactions with the propyl chain were responsible, rather than polar interactions with uncapped silanol groups. This led us to modify the cartridge preparation procedure and the eluting buffers.

PRS-SPE Cartridge Preparation and Eluting Buffer Modifications

See Table 5 for volatile buffer formulations. After thoroughly washing and conditioning the cartridges, care was taken to prevent drying out until after the sample was loaded, as recommended by the manufacturers. However, it was found that separation was enhanced if all of the previous eluant was removed before adding the next, i.e. if the cartridge was momentarily dried between buffers. The volume of new buffer required to change the pH of the cartridge effluent was found to be 2.8mLs for the 500mg and 4.6mLs for the 1g cartridge. Consequently 3mLs and 5mLs were used as working column volumes, respectively, for each cartridge size.

Table 6A

% Hydroxyproline Recovery from PRS-SPE Cartridges v. Loading

Cartridge Size	% of Sorbent Dry Weight Loaded	% Recovery in Each Eluting Buffer pH 1.9	pH 3.1	pH 3.5
500mg	1	0	81	27.5
500mg	3	42.5	57.5	
500mg	5	75	22	
500mg	10	90	12	
500mg	15	90	8	
1g	2	29	67	
1g	4	89	16	
1g	6	95	11	
1g	12	93	5	

(Values are the average of duplicate loadings

Table 6B

% Fluorescent Crosslink Recovery from PRS-SPE Cartridges vs Loading

Cartridge Size	% of Sorbent Dry Weight Loaded	Eluting pH	% Pyridinoline	% Pentoside
500mg	1	4.7	100	100
500mg	3	4.7	100	100
500mg	5	3.5	36	18
		4.7	72	85
500mg	10	3.5	50	11
		4.7	50	89
1g	2	4.7	100	100
1g	4	3.5	33	0
		4.7	67	100
1g	6	3.5	43	6
		4.7	56	94

(Values are the average of duplicate loadings.)

Table 7

Comparison of Intermediate and Mature Collagen Crosslink Quantitation in the Selected Muscles, Expressed as Moles Crosslink / Mole Collagen, following CF1 or PRS-SPE Cartridge Pre-fractionation and Quantitation by either Amino Acid Analysis of RP-HPLC

		Mature X-Link		Intermediate X-Links	
Muscle	**Pre-fract.**	**Pyr. (AAA)**	**Pyr. (RP-HPLC)**	**DHLNL**	**HLNL**
Gastrocnemium	CF1	0.12		0.08	0.07
	SPE	0.23	0.14	0.11	0.06
Ext. carp. rad.	CF1	0.13		0.07	0.03
	SPE	0.13	0.13	0.09	0.06
Flex. hal. long.	CF1	0.15		0.04	0.04
	SPE	0.08	0.16	0.02	0.03
Semitend.	CF1	0.09		0.30	0.70
	SPE	0.16	0.11	0.07	0.60
Psoas maj.	CF1	ND		0.16	0.09
	SPE	0.47	0.02	0.14	0.08
Supraspinatus	SPE	0.16		0.12	0.04
	CF1	0.20	0.15	0.08	0.04
Triceps. brach.	CF1	0.13		0.07	0.06
	SPE	0.26	0.14	0.11	0.05
Pect. prof.	CF1	0.11		0.13	0.09
	SPE	0.09	0.12	0.19	0.12
Latiss. dors.	CF1	0.05		0.18	0.27
	SPE	0.26	0.07	0.17	0.46
Semispin. cap.	CF1	0.08		0.08	0.07
	SPE	0.16	0.10	0.10	0.09

(continued)

Table 7 (Continued)

Comparison of Intermediate and Mature Collagen Crosslink Quantitation in the Selected Muscles, Expressed as Moles Crosslink / Mole Collagen, following CF1 or PRS-SPE Cartridge Pre-fractionation and Quantitation by either Amino Acid Analysis of RPHPLC

		Mature X-Link		Intermediate X-Links	
Muscle	**Pre-fract.**	**Pyr. (AAA)**	**Pyr. (RP, HPLC)**	**DHLNL**	**HLNL**
Serr. vent. thor.	CF1	0.34		0.12	0.08
	SPE	0.16	0.30	0.16	0.06
Splenius	CF1	0.12		0.08	0.07
	SPE	0.23	0.15	0.11	0.06

PRS Cartridge Loading Capacity

Tables 6A and 6B show the influence of increased loading on the binding of hydroxyproline and the fluorescent crosslinks respectively.

As the load increases, so the proportion of hydroxyproline eluting in the loading buffer increases. Loading in excess of 3% of the sorbent dry weight results in approximately half the standard hydroxyproline not being retained on the cartridge in the pH 1.9 loading buffer. This was to be expected as hydroxyproline would be the least strongly adsorbed of the standard mixture. Provided that 3% of the sorbent dry weight or less, is loaded, then the first three eluants can be pooled and assayed to provide a value for percentage collagen in the parent tissue

The data in Table 6B shows the same trend as that in 6A, i.e. larger loadings result in earlier elution from the cartridges. However, it also shows that even if over 3% of the sorbent dry weight is loaded the fluorescent crosslinks do not converge upon the elution position of hydroxyproline.

It is interesting to note, that pyridinoline seems more susceptible to loading affects than pentosidine, presumably reflecting the latters stronger affinity for cation exchange sorbents.

CF1 Pre-fractionation v. PRS Solid Phase Extraction Cartridges.

Table 7 shows how closely, in the majority of cases, the two pre-fractionation methods agree when quantitating the intermediate collagen crosslinks by amino acid analysis and how far they differ when quantitating pyridinoline by the same analytical procedure. However, the quantitation of pyridinoline recovery from the PRS-SPE by RP HPLC, closely agrees with that from the CF1, indicating the presence of co-migrants in the PRS-SPE eluants that led to errors in quantitation by the amino acid analyser.

CONCLUSIONS

Of the 12 solid phase extraction sorbents tested, the propylsulphonic acid (PRS) cartridges proved most effective, being capable of resolving the basic crosslinking amino acids from the majority of acidic and neutral amino acids.

The development of a volatile elution buffer procedure, coupled with the extremely small elution volumes involved (3-5 mLs / eluant), permits rapid further processing after pre-fractionation. In fact, the elution buffer procedure can be further simplified, as the pH 3.1 and pH 6.5 eluants can be dispensed with, provided that sample loading is no more than 3% of the sorbent dry weight. In this case, pyridinoline and pentosidine are retained up to pH 4.7. Any residual material of potential interest can be desorbed from the cartridges using 4N ammonium hydroxide.

The recovery of standard pyridinoline and pentosidine was greater than that reported from either of the other pre-fractionation sorbents, CF1 and SP Sepharose.[7] In addition, the hydroxyproline recovery and resolution from the crosslinking components of a standard mixture have been shown to be consistent within an adequately wide range of sample loadings, for example, 3% of a 500mg cartridge represents a 15mg sample load. In our experience, such a loading would provide sufficient mature and intermediate crosslinks for analysis by the Alpha Plus amino acid analyser of tissues containing as little as 20% collagen. Analysis of pentosidine and pyridinoline by RP HPLC requires less than 300μg collagen, so 15mg of sample would provide sufficient fluorescent crosslinks from a tissue containing as little as 2% collagen. In addition the wide range of SPE cartridge sizes that are commercially available, up to 15g for example, allow the analysis of samples with even lower collagen contents.

The fact that pre-fractionation with PRS-SPE cartridges utilises the same chromatographic principle as one of the subsequent analytical tools, i.e. the amino acid analyser, provides a likely explanation for the poor agreement between the pre-fractionation procedures when assaying pyridinoline by the

Alpha Plus analyser. Presumably, components desorbed from PRS-SPE in the same eluant as the collagen crosslinks will co-elute with them on the amino acid analyser. CF1, on the other hand, exploits the differences in degree of hydrophobicity between the amino acids of interest and, therefore, subsequent amino acid analysis reveals fewer co-migrants. This suggestion is supported by the observation that the intermediate crosslinks, which elute later than pyridinoline from the Alpha Plus analyser, show much closer agreement between the pre-fractionation procedures because few of the PRS-SPE co-migrants are retained on the analytical column as long as they are. RP HPLC on the other hand, utilises a different chromatographic principle to the PRS ion-exchange cartridges and a different detection system to the Alpha Plus analyser for the analysis of pyridinoline, resulting in much closer agreement between the pre-fractionation procedures for pyridinoline quantitation.

The expected variation in intra-muscular collagen crosslinking with variation in muscle growth coefficient was not demonstrable, but the experiment did provide an opportunity to rigorously test the limitations of the PRS-SPE procedure as the collagen content of these whole muscles ranged between 0.8% and 2.2%. The PRS-SPE cartridges proved as reliable and capable of recovering the pyridinoline from the minute amount of collagen in these samples as the generally accepted CF1 pre-fractionation procedure. In addition, we have already demonstrated the superiority of the PRS-SPE cartridge for recovering standard pentosidine over that of the preferred alternative to CF1 for this type of analysis, i.e. SP Sepharose.

Propylsulphonic acid solid phase extraction cartridges are an inexpensive, rapid and reliable pre-fractionation sorbent which, when combined with the volatile organic buffer procedure reported here, provides higher recovery yields of both the mature collagen crosslink pyridinoline and the advanced glycation end-product pentosidine than previously reported. The very small volumes involved during processing and the sample loading capacity makes PRS-SPE a useful tool for subsequent quantitation of these fluorophores by RP HPLC.

REFERENCES

1. A. J. Bailey, "The Chemistry of Natural Enzyme-Induced Cross-Links of Proteins", Amino acids, **1**, 293-306 (1991).

2. D. R. Eyre, T. J. Koot , K. P. Van-Ness, "Quantitation of Hydroxy-pyridinium Crosslinks in Collagen by High Performance Liquid Chromatography, Anal. Biochem., **137**, 380-388 (1984).

3. R. G. Paul, A. J. Bailey, "Glycation of Collagen," Intern. J. Biochem. and Cell Biol, (1996), in press.

4. D. R. Sell, V. M. Monnier, "Structure Elucidation of a Senescence Crosslink from Human Extracellular Matrix-Implication of Pentoses in the Ageing Process, J. Biol. Chem., **264**, 21597-21602 (1989).

5. D. Black, M. Duncan, S. P. Robins, "Quantitative Analysis of the Pyridinium Crosslinks of Collagen in Urine using Ion-paired Reverse Phase High Performance Liquid Chromatography, Anal. Biochem., **169**, 197-203 (1988).

6. T. J. Sims, A. J. Bailey, "Quantitative Analysis of Collagen and Elastin Crosslinks Using a Single-Column System," J.Chrom., **582**, 49-53 (1992).

7. M. Takahashi, T. Ohishi, H. Aoshima, K. Kushida, T. Inoue, K. Horiuchi, "Pre-Fractionation with Cation Exchanger for Determination of Intermolecular Crosslinks, Pyridinoline, and Pentosidine, in Hydrolysate", J. Liq. Chrom., **16(6)**, 1355-1370 (1993).

8. D. R. Sell, V. M. Monnier, "Isolation, Purification and Partial Characterisation of Novel Fluorophores from Aging Human Insoluble Collagen-rich Tissue", Conn. Tiss. Res., **19,** 77-92 (1989).

9. D. G. Dyer, J. A. Blackledge, S. R. Thorpe, J. W. Baynes, "Formation of Pentosidine During Nonenzymatic Browning of Proteins by Glucose," J. Biol. Chem., **268 (18)**, 11654-11660 (1991).

10. R. M. Butterfield, R. T. Berg, "A Classification of Bovine Muscles, Based on their Relative Growth Patterns", Res. Vet. Sci., **7**, 326-332 (1966).

11. N. C. Avery, A. J. Bailey, "An Efficient Method for the Isolation of Intramuscular Collagen, Meat Science," **41(1)**, 97-100 (1995).

12. S. P. Robins, M. Shimokamaki, A. J. Bailey, "The Chemistry of Collagen Crosslinks: Age Related Changes in the Reducible Components of Intact Bovine Collagen Fibres," Biochem. J., **131**, 771-780 (1973).

13. D. W. Bannister, A. B. Burns, "Adaptation of the Bergman & Loxley Technique for Hydroxyproline Determination to the Autoanalyser and its Use in Determining Plasma Hydroxyproline in the Domestic Fowl," Analyst, **95**, 489-496 (1970).

14. A. J. Bailey, T. J. Sims, N. C. Avery, C. A. Miles, "Chemistry of Collagen Crosslinks; Glucose-mediated Covalent Crosslinking of Type IV Collagen in Lens Capsules," Biochem. J., **296**, 489-496 (1993).

15. R. M. C. Dawson, D. C. Elliott, W. H. Elliott, K. M. Jones., In **Data for Biochemical Research**, 3rd edition, Oxford Science Publications, 443 (1991).

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CANONICAL CORRELATION ANALYSIS OF THE RELATIONSHIP BETWEEN PHYSICOCHEMICAL PARAMETERS OF STEROIDAL DRUGS AND THEIR RETENTION CHARACTERISTICS IN HPLC

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ABSTRACT

The retention of 13 steroidal drugs was determined on a polyethylene-coated silica column using methanol-water mixtures at various organic phase concentrations. Good linear relationships were found between the log k' values and the organic mobile phase concentration in the eluent. The slope and intercept values of the relationships were correlated with the physicochemical parameters of the steroidal drugs by means of canonical correlation analysis. Calculation proved that the retention characteristics significantly depend on the physicochemical parameters of the solute. The electronic parameters exerted a higher impact on the retention than the hydrophobicity did. This result, is probably due to the hydrophobic interactions between the polar substructures of drugs and the free silanol groups not covered by the hydrophobic ligand.

INTRODUCTION

Many efforts have been devoted to the elucidation of the relationship between the various physicochemical parameters of solutes and their retention behavior[1-3] in high performance liquid chromatography (HPLC). Various multivariate methods, such as, factor analysis,[4,5] principal component analysis,[6,7] stepwise regression analysis[8] and spectral mapping techniques,[9] have been frequently used for the calculation of various structure-retention relationships.

A more basic understanding of the structure-retention relationships may facilitate optimization processes[10] and the rational selection of stationary phases.[11]

Canonical correlation analysis (CCA) has been developed to find a relationship between two different data matrices.[12] The method of calculation is similar to those of principal component analysis[13] and factor analysis.[14]

Firstly, CCA extracts theoretical factors which explain the maximal variance in the matrix containing the lower number of variables (matrix II, i.e. more than one retention parameters of a set of solutes).

The number of factors explaining 100% of the variance in matrix II is equal to the number of original variables in matrix II. Then, CCA extracts from the other matrix (matrix I, i.e. various physicochemical parameters of the same solutes), variables which have the highest correlation with the factors extracted from matrix II.

Despite its obvious advantages, CCA found only limited application in chromatography. It has been used both in TLC[15] and HPLC,[16,17] for the evaluation of the influence of various physicochemical parameters on the retention behaviour of homologous series of solutes.

A wide variety of HPLC techniques has been developed for the separation and quantitative determination of various steroid drugs.[18-20] However, the relationship between the retention behavior of steroids and their physicochemical parameters has not been studied in detail.

The objectives of our investigation were to determine the retention behavior of some steroidal drugs on a polyethylene-coated silica column, and to elucidate the relationship between retention characteristics and physicochemical parameters of drugs by means of canonical correlation analysis.

Table 1

IUPAC Names of Steroidal Drugs

No. of Drug	IUPAC Name
1	17-Hydroxy-19-norpregn-4-en-3,20-dione
2	11β,17,21-Trihydroxy-19-norpregn-4-en-3,20-dione
3	11β,16α,17,21-Tetrahydroxy-19-norpreng-1,4-dien-3,20-dione-16,17-acetonide
4	17-Hydroxy-19-norpregn-4-en-20-yn-3-one
5	17,21-Dihydroxy-19-norpregn-4-en-3,20-dione
6	7-(Acetylthio)-17-hydroxy-3-oxo-pregn-4-ene-21-carboxylic acid τ-lactone
7	17α-Pregna-2,4-dien-20-yno[2,3-d]isoxazol-17-ol
8	13-Ethyl-17-hydroxy-18,19-donirpregn-4-en-20-yn-3-one
9	17β-Phenylacetyl-17-methylandrosta-1,4-dien-3-one
10	Estra-1,3,5(10)-triene-3,17-diol
11	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diacetate
12	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol
13	3-Acetylestra-1,3,5,(10)-trien-17-one

MATERIALS AND METHODS

The IUPAC names of steroidal drugs are compiled in Table 1. The polyethylene-coated silica support was prepared in our laboratory; its retention characteristics were previously reported.[21] A 25 cm x 4 mm I.D. column was filled with a Shandon Analytical HPLC Packing Pump (Pittsburgh, USA) by the procedure normally used for the filling of reverse phase columns.[22] The HPLC equipment consisted of a Gilson gradient analytical system (GILSON Medical Electronics Villiers-le-Bell, France) with 2 piston pumps (Model 302), Detector (Model 116), Rheodyne injector with 20 μl sample loop (Cotati, California, USA), and a Waters 740 integrator (Milford, Massachusetts, USA). The flow-rate was 1.0 mL/min and the detection wavelength was 225 nm. Mixtures of methanol - water were used as eluents.

Methanol concentration ranged from 40 to 70 vol.% in steps of 5 vol.%. The drugs were dissolved in the eluents at a concentration of 0.05 mg/mL. The average retention time of each compound was determined by three consecutive determinations. Linear correlation was used to describe the dependence of the log k' value on the methanol concentration:

$$\log k' = \log k'_0 + b \cdot C \qquad (1)$$

where $\log k'$ is the logarithm of capacity factor; $\log k'_0$ is the logarithm of capacity factor extrapolated to zero methanol concentration in the eluent (intercept); b is the change of $\log k'$ value caused by unit change (1 vol%) of methanol concentration (slope); and C is the methanol concentration in the eluent (vol %).

To find the molecular parameters significantly influencing the retention, the intercept and slope values of eq.1 were correlated with the physicochemical characteristics of steroidal drugs using canonical correlation analysis. The physicochemical parameters included in the calculation were: π = Hansch - Fujita's substituent constant characterizing hydrophobicity; H - Ac and H - Do = indicator variables for proton acceptor and proton donor properties, respectively; M - RE = molar refractivity; F and R = electronic parameters characterizing the inductive and resonance effects, respectively; σ = Hammett's constant, characterizing the electron-withdrawing power of the substituent; Es = Taft's constant, characterizing steric effects of the substituent; B_1 and B_4 = Sterimol width parameters determined by distance of substituents at their maximum point perpendicular to attachement.

Canonical correlation analysis was carried out on the data matrices consisting of the slope and intercept values of eq.1 (matrix II containing the retention characteristics of steroidal drugs) and of the physicochemical parameters listed above (matrix I).

Calculations were carried out with an IBM AT computer. Canonical correlation analysis software was prepared by Dr. Barna Bordás, Plant Protection Institute of Hungarian Academy of Sciences, Budapest, Hungary.

RESULTS AND DISCUSSION

The parameters of eq.1 are compiled in Table 2. The correlation between the methanol concentration in the eluent and the logarithm of the capacity factor is, in each instance, significant, indicating the regular retention behavior of the solutes.

The slope and intercept values of the steroidal drugs differ considerably, suggesting that the drugs can be sucessfully separated on this column by an appropriate mixture of methanol - water. The values of standard deviation are low, indicating the good reproducibility of the retention time on polyethylene-coated silica column.

Table 2

Parameters of Linear Correlations Between the Logarithim of Capacty Factor and Methanol Concentration in the Eluent.

$$\log k' = \log k'_0 + b \cdot c$$

Compound No.	$\log k'_0$	$-b \cdot 10^2$	$s_b \cdot 10^3$	r_{calc}
1	2.76	4.49	0.9	0.9990
2	1.79	3.81	1.7	0.9953
3	2.26	4.50	1.2	0.9981
4	2.79	4.76	1.13	0.9980
5	2.34	4.35	1.4	0.9973
6	3.07	5.09	2.4	0.9945
7	3.88	5.74	2.25	0.9971
8	2.83	4.69	0.7	0.9995
9	6.37	5.12	0.4	0.9999
10	2.72	4.40	0.8	0.9994
11	4.09	5.34	1.7	0.9990
12	2.90	4.99	1.1	0.9991
13	3.49	5.03	1.5	0.9983

s_b = standard deviation of the slope 'b' value.

The standard and weighted canonical coefficients of the relationships corresponding to the two columns in data matrix II (retention parameters) are compiled in Tables 3 and 4.

The relationships found by CCA indicate that the retention behavior of steroidal drugs on polyethylene-coated silica column considerably depends on the physicochemical parameters included in the calculation. The high r^2 values suggest that this set of physicochemical parameters can be successfully used for the prediction of the retention behavior of steroidal drugs on polyethylene-coated column.

Both chromatographic parameters have similar weights in the second equation (but not in the first one), indicating that these parameters are related but they are not identical. This observation indicates that the information content of both retention parameters is different: therefore, they can be separately included in future structure-retention relationship studies. The

Table 3

Relationship Between the Physicochemical Parameters and Retention Characteristics of Steroidal Drugs. Results of Canonical Correlation Analysis. First Equation

Variables	Canonical Coefficients	
	Standard	Weighted %
Chromatographic Parameters		
$\log k'_0$	-0.31	12.27
b	0.95	87.73
Physicochemical Parameters		
π	-0.53	2.77
H-Ac	1.56	14.69
H-Do	-0.73	11.99
M-RE	-1.02	0.73
F	0.55	21.54
R	0.16	3.67
σ	-0.57	26.22
Es	0.58	1.35
B_1	-1.92	11.34
B_4	1.64	5.70

$r^2 = 0.9824$; $Chi^2 = 35.37$.

impact of the various physicochemical parameters on the retention behavior is similar in both equations. Surprisingly, the calculated molecular hydrophobicities of drugs exert a negligible influence on the retention; however, both the column and the eluents are typical for reverse phase chromatographic system. This result, can be explained by the supposition that the apolar polyethylene chains lie parallel to the silica surface; therefore, the possibility of hydrophobic interactions between the stationary phase and the solutes is lower than in the case of octade-cylsilica columns.

It can be further assumed, that the polyethylene layer does not entirely cover the silica surface, and the hydrophilic interactions occurring between the active adsorption centers not covered by the hydrophobic ligand and the polar substructures of drugs may be involved in the retention mechanism. This assumption, is supported by the finding that the electron-withdrawing power of the substituents, their inductive and resonance effect and proton acceptor capacity have the highest weights in the equations.

Table 4

Relationship Between the Physicochemical Parameters and Retention Characteristics of Steroidal Drugs. Results of Canonical Correlation Analysis. Second Equation

Variables	Canonical Coefficients	
	Standard	Weighted %
Chromatographic Parameters		
$\log k'_0$	0.80	50.59
b	-0.78	49.41
Physicochemical Parameters		
π	0.03	0.31
H-Ac	-1.42	17.47
H-Do	0.70	8.55
M-RE	0.05	0.56
F	-1.47	18.01
R	-1.50	18.39
σ	2.09	25.64
Es	-0.05	0.62
B_1	0.60	7.31
B_4	-0.26	3.14

r^2 = o.9068; Chi^2 = 13.05

The results of canonical correlation analysis are supported by the fact that some significant correlations were found between the individual retention characteristics and the physicochemical parameters having high weights in the equations (n = 13; $r_{95\%}$ = 0.5529):

$$\log k'_0 = 2.51 - (1.26 \pm 0.34) \cdot R \qquad r_{calc} = 0.7492 \qquad (2)$$

$$b = 4.55 - (0.46 \pm 0.17) \cdot R \qquad r_{calc} = 0.6329 \qquad (3)$$

$$b = 5.48 - (0.91 \pm 0.36) \cdot \sigma \qquad r_{calc} = 0.6075 \qquad (4)$$

The canonical variates calculated from matrix II (related to the relative importance of the individual steroidal drugs in the determination of the retention parameters) are compiled in Table 5. The data in Table 5 clearly show that the contributions of the drugs to the determination of the

Table 5

Contribution of Individual Steroidal Drugs to the Relationships Between Retention Characteristics and Physicochemical Parameters. Canonical Variates Calculated from Matrix II.

No. of Drugs	First Equation	Second Equation
1	-0.46	-0.09
2	-1.51	-0.33
3	-0.32	-0.50
4	-0.03	-0.28
5	-0.62	-0.32
6	0.60	-0.36
7	1.62	-0.17
8	-0.10	-0.19
9	-0.24	2.31
10	-0.62	-0.06
11	0.80	0.31
12	0.45	0.38
13	0.37	0.07

chromatographic parameters is highly different. We assume that similar data can be used for the selection of solute sets - with the number of solutes as low as possible-which are suitable for characterisation of chromatographic systems.

It can be concluded from the results, that canonical correlation analysis is a suitable method for the assessment of the relationships between the retention behavior of steroidal drugs on polyethylene-coated silica support and their physicochemical parameters. Calculation indicated, that the retention characteristics of this support differ from that of traditional octadecylsilica support and the electronic parameters of drugs exert a considerable influence on the retention, suggesting the involvement of hydrophilic forces in the solute - support interaction.

REFERENCES

1. R. Kaliszan, "Quantitative Structure-Retention Relationships Applied to Reverse Phase High Performance Liquid Chromatography, " J. Chromatogr. A, **656**, 417 (1993).

2. R. M. Smith, "Functional Group Contributions to the Retention of Analytes in Reverse Phase High Performance Liquid Chromatography," Chromatogr. A, **656**, 381, (1993).

3. N. Chen, Y. Zhang, P. Lu, "Effects of Molecular Structure on the Log k'_w Index and Linear S-log k'_w Correlation in Reverse Phase High Performance Liquid Chromatography," J. Chromatogr., **633**, 31, (1993).

4. M. Righezza, J. R. Chretien, "Factor Analysis of Experimental Design in Chromatography," Chromatographia, **36**, 125, (1993).

5. U. Haldna, J. Pentchuk, M. Righezza, J. R. Chretien, "Factor Analysis in Ion Chromatography of Carboxylate Ions," J. Chromatogr. A, **670**, 51, (1994).

6. P. Karsnas, T. Lindblom, "Characterization of Hydrophobic Interaction and Hydrophobic Interaction Chromatography Media by Multivariate Analysis," J. Chromatogr., **599**, 131, (1992).

7. R. Gami-Yilinkou, A. Nasal, R. Kaliszan, "Application of Chemometrically Processed Chromatographic Data for Pharmacological Relevant Classification of Antihistamine Drugs," J. Chromatogr., **633**, 57, (1993).

8. R. Kaliszan, K. Osmialowski, "Correlation Between Chemical Structure of Non-Congeneric Solutes and their Retention on Polybutadiene-Coated Alumina," J. Chromatogr., **506**, 3, (1990).

9. T. Hamoir, F. Cuesta Sanchez, B. Bourgignon, D. L. Massaart, "Spectral Mapping Analysis: A Method for the Characterization of Stationary Phases," J. Chromatogr. Sci., **32**, 488, (1994).

10. J. R. Torres-Lapasió, R. M. Villanueva-Camanas, J. M. Sanchis-Mallols, M. J. Medina-Hernandez, M. C. Garcia-Alvarez-Coque, "Interpretative Strategy for Optimization of Surfactant and Alcohol Concentration in Micellar Liquid Chromatography," J.Chromatogr. A, **677**, 239, (1994).

11. R. J. M. Vervoort, M. W. J. Derksen, F. A. Maris, "Selection of Stationary Phases for the Liquid Chromatographic Analysis of Basic Compounds Using Chemometric Methods," J. Chromatogr. A, **678**, 1, (1994).

12. L. Orloci, C. R. Rao, W. M. Stitiler, **Multivariate Methods in Ecological Work**, International Cooperative Publishing House, Fairland, Maryland, USA, 1979.

13. K. V. Mardia, J. T. Kent, J. M. Bibby, **Multivariate Analysis**, Academic Press, London, 1979.

14. E. R. Malinowski, D. C. Howery, **Factor Analysis in Chemistry**, Wiley, New York, 1980.

15. T. Cserháti, E. Forgács, "Use of Canonical Correlation Analysis for the Evaluation of Chromatographic Retention Data," Chemom. Int. Lab. Syst., **28**, 305, (1995).

16. E. Forgács, T. Cserháti, B. Bordás, "Application of Multivariate Mathematical-Statistical Methods for the Comparison of the Retention Behaviour of Porous Graphitized Carbon and Octadecyl-Silica Columns," Anal. Chim. Acta, **279**, 115, (1993).

17. E. Forgács, T. Cserháti, B. Bordás, "Comparison of the Retention Behaviour of Phenol Derivatives on Porous Graphitized Carbon and Octadecyl-Silica Columns," Chromatographia, **36**, 19, (1993).

18. J. Novakovic, E. Tvrzicka, V. Pacakova, "High-Performance Liquid Chromatographic Determination of Equine Estrogens with Ultraviolet Absorbance and Electrochemical Detection," J. Chromatogr. A, **678**, 359, (1994).

19. J. Lykkesfeldt, S. Loft, H. E. Poulsen, "Simultaneous Determination of Urinary Free Cortisol and 6ß-Hydroxycortisol by High Performance Liquid Chromatography to Measure Human CYP3A Activity," J. Chromatogr. B, **660**, 23, (1994).

20. S. M. R. Stanley, B. S. Wilhelmi, J. P., Rodger, A. Guthrie, "Detection of Corticosteroids by High Performance Liquid Chromatography/Negative Ion Chemical Ionization Mass Spectrometry Using a Particle Beam Interface," Biol. Mass Spectrom., **23**, 483, (1994).

21. E. Forgács, "Retention Behavior of Some Ring-Substituted Aniline Derivatives on Polyethylene-Coated and Octadecylsilica Columns," J. Liquid Chromatogr., **16**, 3757, (1993).

22. **Instruction Manual HPLC Packing Pump,** Shandon, Cheshire, UK, 1989.

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AUGUST 11 - 15: 26th ACS Northeast Regional Meeting, Western Conn State Univ, Danbury, CT. Contact: A. Alder, 11 Long Ridge Rd, Redding, CT 06896, USA; (203) 938-2920; Email: reglmtgs@acs.org.

AUGUST 11 - 16: 3rd International Hydrocolloids Conference, Sydney, Australia. Contact: Gail Hawke, P. O. Box N-399, Grosvenor Place, Sydney, NSW 2000, Australia. Tel: 61 02 241 3388; FAX: 61 02 241 5282.

AUGUST 11 - 16: ICORS '96: 15th International Conference on Raman Spectroscopy, Pittsburg, Pennsylvania. Contact: Sanford Asher, Chem Dept, University of Pittsburgh, PA 15260, USA. Tel: (412) 624-8570.

AUGUST 12 - 16: 11th Internationa Congress on Thermal Analysis & Calorimetry, Philadelphia. Contact: The Complete Conference, 1540 River Pk Dr, Sacramento, CA 95815, USA. Tel: (916) 922-7032.

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: 17th International Conference on Magnetic Resonance in Biological Systems, Keystone, Colorado, USA. Contact: Conference Office, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4735; FAX: (505) 989-1073.

AUGUST 21 - 23: 4th International Symposium on Capillary Electrophoresis, York, UK. Contact: Dr. T. Threlfall, Industrial Liaison Executive, Dept of Chem, University of York, Heslington, York, YO1 5DD, UK.

AUGUST 25 - 29: 212th ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; Email: natlmtgs@acs,org.

AUGUST 25 - 30: International Symposium on Metal Hydrogen Systems: Fundamentals and Applications, Les Diablerets, Switzerland. Contact: MH-96, Inst of Physics, Univ of Fribourg, Perolles, CH-1700 Fribourg, Switzerland. Tel: 41 37 299 113; FAX: 41 37 299 772.

AUGUST 25 - 30: 12th International Congress on Chemical & Process Engineering, Praha, Czech Republic. Contact: Organizing Committe, CHISA'96, P. O. Box 857, 111 21 Praha, Czech Republic. Tel: 42 2 353287; FAX: 42 2 3116138.

SEPTEMBER 1 - 4: 4th Inetrnational Symposium on Preparative & Industrial Chromatography & Related Techniques, Basel, Switzerland. Contact: Secretariat Prep'96, Messeplatz 25, CH-4021 Basel, Switzerland. Tel: 41 61 686 28 28; FAX: 41 61 686 21 85.

SEPTEMBER 1 - 6: IUPAC Chemrawn IX, Seoul, Korea. Contact: IUPAC Chemrawn IX, Secretariat, Tongwon B/D 6th Floor, 128-27 Tangjudong, Chongro-ku, Seoul 110-071, Korea. FAX: 82 2 739-6187.

SEPTEMBER 1 - 6: 11th International Symposium on Organosilicon Chemistry, Monpellier, France. Contact: R. Corriu, University of Montpellier II, Place Eugene Batallon, cc007, 34095 Montpellier cedex 05, France. Tel: 67 14 38 01.

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.

SEPTEMBER 4 - 9: International "Thermophiles '96" Symposium: Biology, Ecology & Biotechnology of Thermophilic Organisms, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

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. Tel: 39 532 291154; FAX: 39 532 240709.

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. Tel: 39 532 291154; FAX: 39 532 240709.

SEPTEMBER 9 - 11: 110th AOAC International Annual Meeting & Expo, Hyatt, Orlando, Florida. Contact: AOAC International, 2200 Wilson Blvd, Suite 400, Arlington, VA 22201-3301, USA. Tel: (703) 522-3032; FAX: (703) 522-5468.

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 10 - 12: International Thermophiles Workshop: Keys to Molecular Evolution & the Origin of Life, Athens, Georgia. Contact: J. Wiegel, University of Georgia, Microbiology Department, 527 Biol Sci Bldg, Athens, GA 30602-2605, USA. Tel: 706) 542-2674; FAX: (706) 542-2651.

SEPTEMBER 11 - 13: Corn Refiners Assn Scientific Conference, Bloomindale, Illinois. Contact: Corn Refiners Assn, 1701 Pennsylvania Ave, NW, Washington, DC 20036, USA. Tel: (202) 331-1634; FAX: (202) 331-2054.

SEPTEMBER 11 - 13: 2nd Workshop on Biosensors & Biol Techniques in Environmental Analysis, Lund, Sweden. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland.

SEPTEMBER 15 - 20: 21st International Symposium on Chromatography, Stuttgart, Germany. Contact: Dr. L. Kiessling, Geselleschaft Deutscher Chemiker, Postfach 900440, D-60444 Frankfurt/Main, Germany.

SEPTEMBER 16 - 18: 3rd International Conference on Applications of Magnetic Resonance in Food Science, Nantes, France. Contact: Laboratoire de RMN-RC, 2 rue de la Houssiniere, 44072 Nantes cedex 03, France. Tel: 33 40 74 98 06; FAX: 33 40 37 31 69.

SEPTEMBER 16 - 19: International Ion Chromatography Symposium 1996, University of Reading, Reading, UK. Contact: J. R. Strimaitis, Century International, P. O. Box 493, Medfield, MA 02052, USA. Tel: (508) 359-8777; FAX: (508) 359-8778.

SEPTEMBER 17 - 20: 10th International Symposium on Cap[illary Electrophoresis, Prague, Czech Republic. Contact: Dr. B. Gas, Dept of Physical Chem, Charles University, Albertov 2030, CZ-128 40 Prague 2, Czech Republic.

SEPTEMBER 19 - 21: 19th Annual Gulf Coast Chemistry Conference, Pensacola, Florida. Contact: J. Gurst, Chem Dept, University of West Florida, Pensacola, FL 32514, USA. Tel: (904) 474-2744; FAX: 904) 474-2621.

SEPTEMBER 20 - 24: 12th Asilomar Conference on Mass Spectrometry, Pacific Grove, California. Contact: ASMS, 1201 Don Diego Ave, Santa Fe, NM 87505, USA. Tel: (505) 989-4517; FAX: (505) 989-1073.

SEPTEMBER 28 - OCTOBER 5: Federation of Analytical Chem & Spectroscopy Societies (FACSS), Kansas City. Contact: J.A. Brown, FACSS, 198 Thomas Johnson Dr., Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 17 - 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.

OCTOBER 27 - 31: American Assn of Pharmaceutical Scientists Annual Meeting & Expo, Seattle, Washington. Contact: AAPS, 1650 King St, Alexandria, VA 22314-2747, USA. Tel: (703) 548-3000; FAX: (703) 684-7349.

OCTOBER 29 - 30: ASTM Symposium on Pesticide Formulations & Application Systems, New Orleans, Louisiana. Contact: G. R. Goss, Oil-Dri Corp, 777 Forest Edge Dr, Vrenon Hills, IL 60061, USA. Tel: (708) 634-3090; FAX: (708) 634-4595.

OCTOBER 29 - 31: Cphl Pharmaceutical Ingredients Worldwide '96 Conference, Milan, Italy. Contact: Miller Freeman BV, Industrieweg 54, P. O. Box 200, 3600 AE Maarssen, The Netherlands. Tel: 31 3465 73777; FAX: 31 3465 73811.

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 6 - 9: 24th Biennial International Conference on Application of Accelerators in Research & Industry, Denton, Texas. Contact: J. L. Duggan or B. Stippec, University of North Texas, Physics Department, Denton, TX 76203, USA. Tel: (817) 565-3252; FAX: (817) 565-2227.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Hyatt Regency Hotel, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

NOVEMBER 10 - 13: 4th North American Research Conference on Organic Coatings Science & Technology, Hilton Head, South Carolina. Contact: A. V. Patsis, SUNY, New Paltz, NY 12561, USA. Tel: (914) 255-0757; FAX: (914) 255-0978.

NOVEMBER 10 - 14: 10th International Forum on Electrolysis in the Chemical Industry, Clearwater Beach, Florida. Contact: P. Kluczynski, Electrosynthesis Co, 72 Ward Road, Lancaster, NY 14086, USA. Tel: (716) 684-0513; FAX: (716) 684-0511.

NOVEMBER 10 - 15: AIChE Annual Meeting, Palmer House, Chicago, Illinois. Contact: AIChE, 345 East 47th Street, New York, NY 10017-2395, USA.

NOVEMBER 11 - 20: 2nd Latin-American Conference on Biomedical, Biopharmaceutical and Industrial Applications of Capillary Electrophoresis, Santiago, Chile. Dr. E. Guerrero, Servicio Medico Legal, Avenida de la Paz 1012, Santiago, Chile.

NOVEMBER 12 - 14: Plastics Fair, Charlotte, North Carolina. Contact: Becky Lerew, Plastics Fair, 7500 Old Oak Blvd, Cleveland, OH 44130, USA. Tel: (216) 826-2844; FAX: (216) 826-2801.

NOVEMBER 13 - 15: 13th Montreux Symposium on Liquid Chromatography-Mass Spectrometry (LC/MS; SFC/MS; CE/MS; MS/MS), Maison des Congres, Montreux, Switzerland. Contact: M. Frei-Hausler, Postfach 46, CH-4123 Allschwill 2, Switzerland. Tel: 41-61-4812789; FAX: 41-61-4820805.

NOVEMBER 18 - 20: 3rd International Conference on Chemistry in Industry, Bahrain, Saudi Arabia. Contact: Chem in Industry Conf, P. O. Box 1723, Dhahran 31311, Saudi Arabia. Tel: 966 3 867 4409; FAX: 966 3 876 2812.

NOVEMBER 17 - 22: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S., Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

NOVEMBER 24 - 27: Industrial Research for the 21st Century, 1996 Biennial Meeting, Red Lion Resort, Santa Barbara, California. Contact: R. Ikeda, DuPont Central Research Dept., P. O. Box 80356, Wilmington, DE 19880-0356, USA. Tel: (302) 695-4382; FAX: (302) 695-8207; Email: ikeda@esvax.dnet.dupont.com.

DECEMBER 17 - 20: First International Symposium on Capillary Electrophoresis for Asia-Pacific, Hong Kong. Contact: Dr. S. F. Y. Li, Dept of Chemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260, Republic of Singapore.

1997

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene 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.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow, Dow Corning Corp., CO42A1, Midland, MI 48686-0994, USA. Tel: (517) 496-6491; FAX: (517) 496-6824.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce, Rust Envir & Infrastructure, 12 Metro Park Rd, Albany, NY 12205, USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept, Wayne State Univ., Detroit, MI 48202-3489, USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown, FACSS, 198 Thomas Johnson Dr, Suite S-2, Frederick, MD 21702, USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal, Chem Dept, Virginia Tech, Blacksburg, VA 24061, USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler, 8340 Luxor St, Downey, CA 90241, USA. Tel: (310) 869-9838; Email: reglmtgs@acs.ord.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler, IAEAC Secretariat, Postfach 46, CH-4123 Allschwill 2, Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128; Email: miscmtgs@acs.org.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218; FAX: (302) 738-5275.

1998

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia

Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw Triangle Pk, North Carolina. Contact: B. Switzer, Chem Dept, N Carolina State University, Box 8204, Raleigh, NC 27695-8204, USA. Tel: (919) 775-0800, ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle, Chem Dept, University of Tennessee, Knoxville, TN 37996, USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC

20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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   Use Times Roman 12 point font, if possible.

The general organization of the manuscript should be:

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Author(s)' names and full addresses
Abstract
Text Discussion
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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

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MATERIALS
METHODS
RESULTS
DISCUSSION
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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.

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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.

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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.**

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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.

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