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DISPLACEMENT THIN-LAYER CHROMATOGRAPHY OF SOME ECDYSTEROIDS

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ABSTRACT

The essential steps of displacement thin-layer chromatography and its applications for separation of ecdysteroids are outlined. Finding adequate conditions of displacement thin-layer chromatography for plant ecdysteroids is detailed including the optimisation of mobile phase composition, mobile phase flow rate, and preelution before displacement development. Application of preelution before displacement chromatography has an importance in the case of planar chromatography, both exploring and achieving the displacement separations by HPLC.

INTRODUCTION

Chromatography with elution type of developments is generally performed at the linear parts of the Langmuir isotherms, while displacement chromatography operates with high load, that is at concentrations that are at the non-linear parts of the isotherms [1].

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Displacement chromatography has served to improve both analytical and preparative separations. Modern variations of chromatography with displacement type of developments include high performance (column liquid) displacement chromatography [1, 2] and planar (thin-layer) displacement chromatography [3, 4].

Using displacement chromatography, there are two mobile phases. One of them is the carrier which is adequate for non-movement (or very slow movement) of the compounds to be purified. The other one is the displacer, that is displacing the sample components from the stationary phase, therefore, pushing them forward. In the case of displacement thin-layer chromatography (D-TLC), the displacer is dissolved in the carrier. There are two fronts of the mobile phase running forward, the carrier front (first front) and the displacer front (the second one). The sample components to be purified have to move in front of the displacer. As the thin-layer chromatogram has been developed, the displaced component forms a well defined, very sharp zone before the displacer front, it can be easily detected and removed for preparative purposes [4].

Ecdysteroids are insect hormones found in insects, in various other animals and also in plants. In insects, ecdysteroids are moulting hormones, in plants their role is not well explained. However, some plants can be the raw materials for the isolation of ecdysteroids because of their high concentration (up to 3.3% which is much higher than in insects). Among several other types of organic compounds (amines, phenyl alkyl compounds, steroids, etc.), ecdysteroids have also been the subject of our displacement chromatographic separations using planar arrangements of the stationary phase [5-8].

New methods for displacement thin-layer chromatography of plant ecdysteroids have been recently developed [6, 7]. In this paper, optimization of conditions of displacement development by using various carriers, displacers, as well as multiple developments will be detailed.

MATERIALS AND METHODS

Pre-coated TLC plates silica gel 60 F-254 (Merck, Darmstadt, Germany), solvents and chemicals from commercial sources were used.

2-Deoxy-20-hydroxyecdysone (**db**) and 20-hydroxyecdysone (**b**) were the kind gift of Dr. D. H. S. Horn (Acherone, Victoria, Australia). The extraction of *Silene otites (L.) Wib.* (**ex**), and the isolation of 2-deoxyecdysone (**a**), 20-hydroxyecdysone-22-acetate (**ac**) and integristerone (**i**) have been described elsewhere [9].

TLC plates were developed in Desaga (Heidelberg, Germany) chambers using non-saturated vapour phase. Solvent systems are detailed in Table 1.

Chrompres 10 (forced-flow TLC equipment) was purchased from Laberte (Budapest, Hungary).

TABLE 1.

SOLVENT SYSTEMS FOR DEVELOPMENT OF TLC PLATES

Solvent system	Ratio
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(220:20:5)
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(160:20:5)
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(140:20:5)
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(140:30:5)
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(110:40:5)
dichloromethane--i.propanol--3-dimethylaminopropylamine (dkm--i.PrOH-DAPA)	(80:30:5)
dichloromethane--i.propanol (dkm--i.PrOH)	(140:20)
ethyl acetate--methanol--ammonia (EtAc--MeOH--NH_3)	(85:10:5)

RESULTS

Fig. 1 presents the alteration of displacement chromatogram when the ratio of dichloromethane was changed from 140:20 to 110:40, and thereby the 2-deoxy-20-hydroxyecdysone left the displacement front and became eluted by the carrier itself, however, the 20-hydroxyecdysone became part of the displacement train. For comparison, the extract of *Silene otites (L.) Wib.* was also spotted, that extract contained both 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone.

Preelution before displacement TLC can improve the separation. Fig. 2 shows the TLC chromatogram after preelution but before displacement (left side) and after performing the displacement separation (right side). With preelution, 20-hydroxyecdysone can be well separated from the overwhelming majority of other components of *Silene otites (L.) Wib.* extract, including the removal of 2-deoxy-20-hydroxyecdysone.

Other arrangements, such as using dichloromethane--i.propanol-3-dimethylaminopropylamine (220:20:5) ratio makes possible the selective concentration of 2-deoxy-20-hydroxyecdysone in the displacement train, as it is demonstrated in Fig. 3.

Also, preelution with ethyl acetate--methanol--ammonia (85:10:5) followed with displacement chromatography (using dichloromethane--i.propanol--3-dimethylaminopropylamine (160:20:10)) makes possible the concentration of both 2-deoxy-20-hydroxyecdysone and 20-hydroxy-ecdysone in the displacement train (Fig. 4.).

Results of displacement thin-layer chromatography with forced-flow developments depend on the flow rate of the mobile phase. This phenomenon is given in Figs. 5 and 6 where the plates were developed with 0.7 and 0.45 ml/min flow rate, thereby both the eluent and the displacer fronts showed peculiar characteristics.

DISCUSSION

Although preparative separation of ecdysteroids is generally done by a combination of various chromatographic procedures [9], efforts have

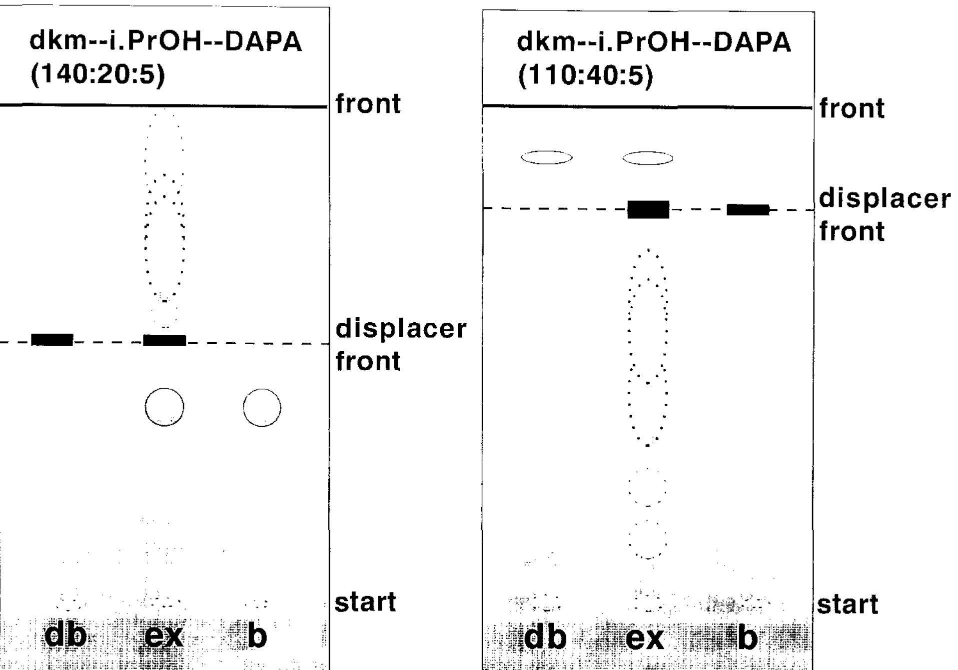

Figure 1.
Displacement thin-layer chromatography of 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone (**b**) and the extract of Silene otites (L.) (**ex**) is given on TLC plates silica gel 60 F-254 using the mobile phases such as dichloromethane--i.propanol--3-dimethylaminopropylamine (140:20:5) and dichloromethane--i.propanol--3-dimethylaminopropylamine (110:40:5) on the left side plate and on the right side plate, respectively.

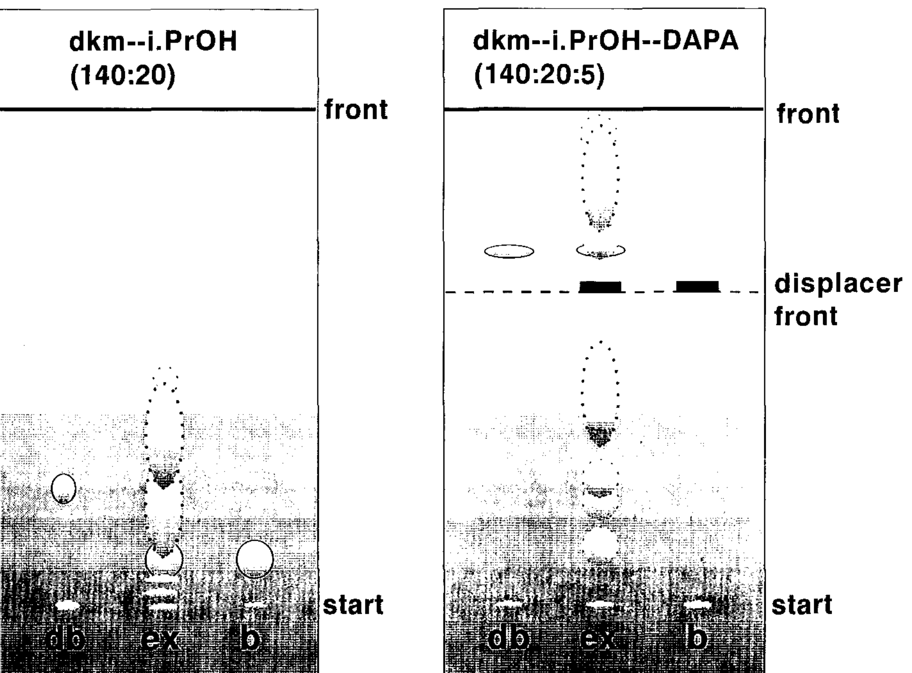

Figure 2.
Thin-layer chromatography followed by displacement thin-layer chromatography of 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone (**b**) and the extract of Silene otites (L.) (**ex**) is given on TLC plate silica gel 60 F-254 using the mobile phases such as dichloromethane--i.propanol (140:20) and dichloromethane--i.propanol--3-dimethylamino-propylamine (140:20:5) on the left side plate and on the right side plate, respectively.

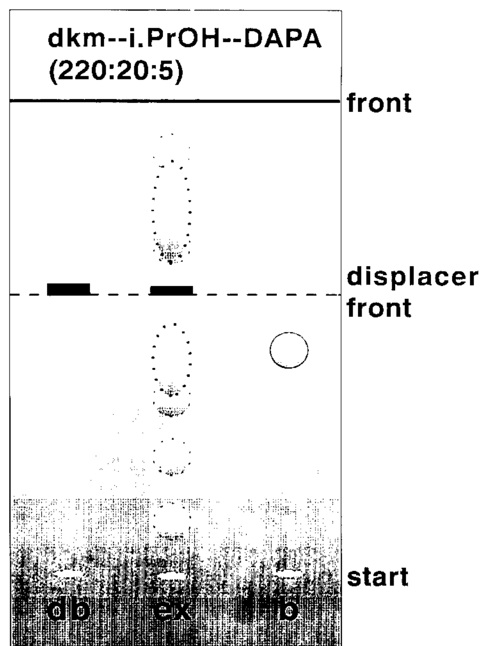

Figure 3.
Displacement thin-layer chromatography of 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone (**b**) and the extract of Silene otites (L.) (**ex**) is given on TLC plate silica gel 60 F-254 using the mobile phase dichloromethane--i.propanol--3-dimethylaminopropylamine (220:20:5).

been made to circumvent the difficulties of the multistep separations. One of these methods is the high-performance displacement chromatography [1] and its variation, the displacement thin-layer chromatography [2-8]. Elution-type developments work with concentrations where the so called adsorption isotherms are linear, thereby the load is limited. At the same time, displacement chromatography works at higher concentration (several mg/ml) which allows the separation of amines, amino acids, peptides, proteins, steroids, generally with good yield.

Displacement thin-layer chromatography of ecdysteroids have been described in our earlier publications, when the influence of the saturation of the chamber and other conditions were investigated [6, 7].

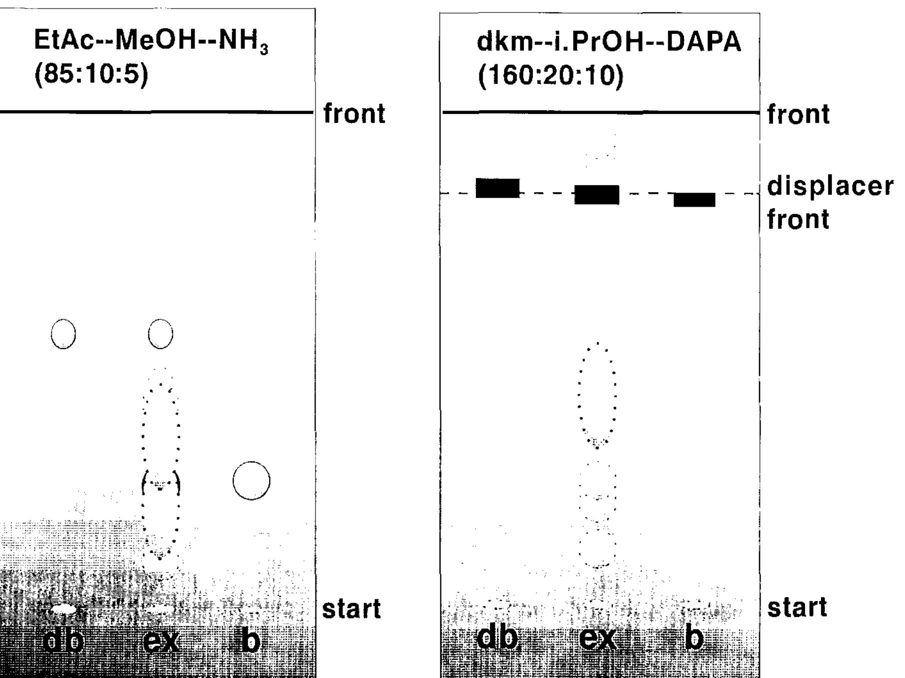

Figure 4.
Thin-layer chromatography followed by displacement thin-layer chromatography of 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone (**b**) and the extract of Silene otites (L.) (**ex**) is given on TLC plate silica gel 60 F-254 using the mobile phases such as ethyl acetate--methanol--ammonia (85:10:5) and dichloromethane--i.propanol--3-dimethylaminopropylamine (160:20:10) are given on the left side and on the right side, respectively.

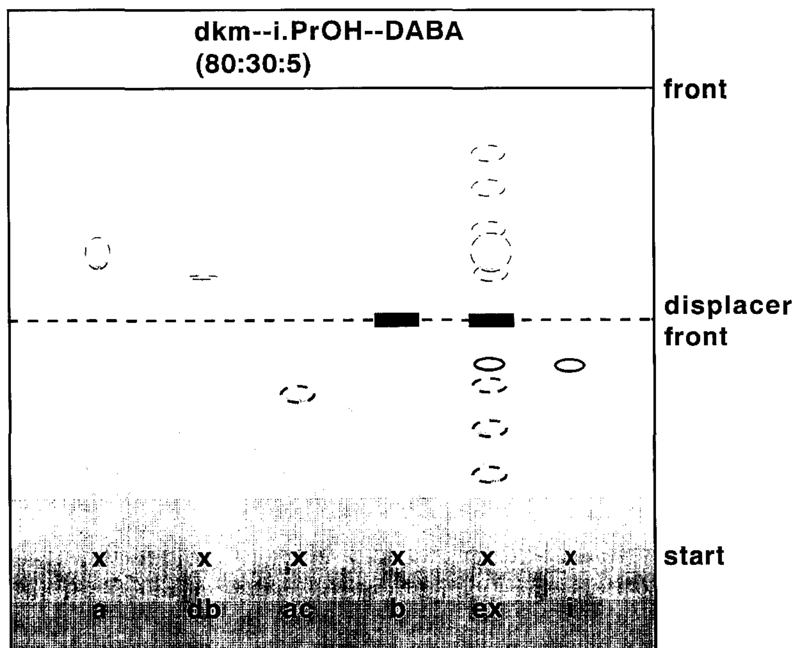

Figure 5.
Forced-flow displacement thin-layer chromatography of 2-deoxyecdysone (**a**), 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone-22-acetate (**ac**), 20-hydroxyecdysone (**b**), the extraction of Silene otites (L.) (**ex**), and integristerone (**i**) is given on TLC plate silica gel 60 F-254 using the mobile phase dichloromethane--i.propanol--3-dimethylaminopropylamine (80:30:5) with flow rate of 0.7 ml/min.

Effective separations were found when two-dimensional (elution-displacement) chromatography was used.

In this paper displacement separations are described, when the preelution and displacement chromatography are performed in the same direction, but elution-type of development precedes displacement, thereby, effective removal of contaminants is possible. Moreover, preelution also influences the development of displacement train. The composition of the system, used for preelution, also determines the

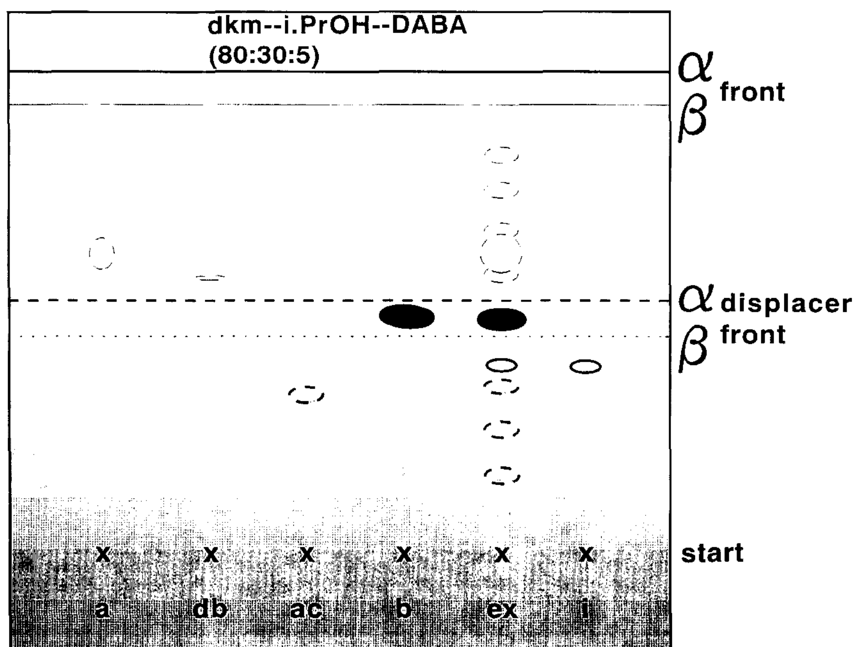

Figure 6.
Forced-flow displacement thin-layer chromatography of 2-deoxyecdysone (**a**), 2-deoxy-20-hydroxyecdysone (**db**), 20-hydroxyecdysone-22-acetate (**ac**), 20-hydroxyecdysone (**b**), the extraction of Silene otites (L.) (**ex**), and integristerone (**i**) is given on TLC plate silica gel 60 F-254 using the mobile phase dichloromethane--i.propanol--3-dimethylaminopropylamine (80:30:5) with flow rate of 0.45 ml/min.

members of the displacement train. These results can give the basis of ecdysteroid separations by displacement HPLC, as has been shown before [6, 9, 11-13].

Thin-layer displacement chromatography can also be performed in a forced-flow system [7]. While the movement of developing solvents in classical planar chromatography is propagated by capillary forces, forced-flow TLC uses pumps to deliver the mobile phase. Thereby, the speed of development can be regulated [7, 10, 11], just as it has been done in the

case of HPLC. For optimal separations, the flow rate should be chosen over a certain limit (Figs. 5, 6). The displacement thin-layer chromatography requires a definite speed of development to reach optimised and reproducible separations.

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QUANTITATIVE THIN LAYER CHROMATOGRAPHY OF INDOLE ALKALOIDS. II. CATHARANTHINE AND VINDOLINE

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ABSTRACT

A method is described for the densitometric determination of the indole alkaloids catharanthine and vindoline in plant extract. The alkaloids were separated from each other and from the rest of the components by three-fold development with a mobile phase of petroleum ether / ethyl ether / acetone / ethanol (70+10+20+1, v/v/v/v). Catharanthine was scanned at 280 nm and vindoline - at 310 nm. The calibration curves were linear in the interval 1 µg/ per spot - 5 µg/per spot. A standard deviation of less than 0.1 µg per spot and a relative error not exceeding 3% were found.

INTRODUCTION

Until recently, the plant *Catharanthus roseus* (L.) G. Don has been the only natural source for the production of the medicinally important binary indole alkaloids vinblastine and vincristine. Unfortunately, the content of these two alkaloids in the plant is very

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low, causing serious complications in their isolation. Obviously these factors influence unfavourably the cost of the final product [1]. The high clinical effect of the vinblastine and vincristine as antitumour agents on one hand and their limited availability on the other provoked an increasing scientific interest in their synthesis. A great number of vinblastine analogues has been synthesized. Some of them have been applied in the chemotherapy of cancer [2, 3]. The alkaloids catharanthine (I) and vindoline (II), Figure 1, have been preferably used as starting materials in these syntheses. They are also present in *Catharanthus roseus* , in amounts exceeding 10 - 40 times that of vinblastine and vincristine [4]. Reliable analytical methods are required for the evaluation of the analytical purity of I and II as well as for the determination of their content in the crude plant material and in the appropriate reaction mixture. Different

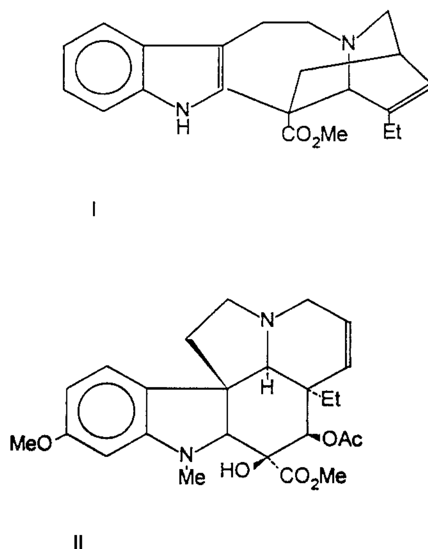

FIGURE 1. The chemical structure of the indole alkaloids catharanthine (I) and vindoline (II)

procedures and techniques have been proposed for the quantitation of indole alkaloids and particularly those of *Catharanthus roseus* [4], among them HPLC being rather advantageous [5].

The experience gained in this laboratory in the analysis of *Vinca* alkaloids has shown that TLC densitometric analysis combined with suitable pre treatment of the sample could be successfully applied to a routine simultaneous determination of the indole alkaloids tabersonine and 11-methoxytabersonine in plant extracts [6].

Presently, we reported about a simplified analytical TLC method for routine densitometric quantitation of catharanthine and vindoline.

EXPERIMENTAL

Materials

All reagents and solvents were of analytical grade and were used without further purification. Petroleum ether had b. p. 40-60 ^{o}C.

20 x 20 cm precoated glass TLC plates Silica gel 60 F_{254} (Merck Art. 5715) were used.

Catharanthine and vindoline were isolated in this laboratory from leaves of *Catharanthus roseus* and purified by column chromatography on aluminium oxide 90, Brockmann II-III, 0.063 0.200 mm (Merck, Art. 1097). The crude catharanthine was recrystallized in acetone, while vindoline in ethyl ether. The purity of the isolated material was checked by TLC, melting point, UV, NMR, IR spectroscopy and mass spectrometry.

Stock solutions of the pure alkaloids were prepared by dissolving 12.88 mg of catharanthine and 12.80 mg of vindoline, respectively in abs. ethanol in 25 ml volumetric flasks. Five working solutions of each alkaloid with concentrations in the range 0.512-0.103 mg/ml catharanthine and 0.512-0.1024 mg/ml vindoline, were prepared by dilution and used to plot the calibration graphs.

Isolation of Total Alkaloids from *Catharanthus roseus*

The finely ground dry plant material (4 g) was extracted repeatedly with five portions of 10 ml each methanol 2N citric acid (1:1, v/v). Duration of each extraction was 30 min. The combined extracts (50 ml) were concentrated under vacuum to a volume of 10 ml and the solution adjusted with ammonia to pH 8-9. The alkaloids were then extracted with 5 successive 15 ml portions of ethyl acetate. The combined extracts were washed with 3 ml of distilled water, dried over anhydrous sodium sulphate and evaporated to dryness (vacuum evaporator, 35° C). The residue was dissolved in abs. ethanol (5 ml) and solvent evaporated as described above. The resulting residue was dried at 40° C under vacuum for 1h, the residue was weighed, transferred quantitatively into a 25 ml volumetric flask and brought to volume with abs. ethanol. 10 μl aliquots of this solution were subjected to chromatography.

Thin Layer Chromatography.

The absorbent layer was cut into two equal parts measuring 20 cm x 10 cm, a double number of samples can be applied on the two opposite wide sides of the plate.

The samples (10 μl aliquots) were applied along a straight line 15 mm above the rim of the plate as spots with diameter not exceeding 6 mm. Spots were spaced at a distance exactly 15 mm from one another. The marginal spots were spaced at a distance of 11 mm from the side edges of the plate.

The plate were then allowed to stay in dark for 15 min to ensure the evaporation of the solvent from the spots. During that time the chromatographic chamber (standard 215 mm x 205 mm x 125 mm) was saturated with the vapours of the mobile phase petroleum ether ethyl ether acetone ethanol (70+10+20+1, v/v). The plates were developed to a solvent front of 85 mm. They were then removed from

the chamber, flushed with cold air for 1 min and allowed to stay in dark at room temperature for 30 min. The developing procedure was repeated three times.

Apparatus

Densitometric measurements were performed on Shimadzu CS 930 dual wavelength scanner equipped with Shimadzu DR 2 data recorder , in zigzag refection mode with a slit size of 1.2 mm x 1.2 mm.

RESULTS AND DISCUSSION.

Thin Layer Chromatography

It is well known that the extracts containing alkaloids isolated from a plant material comprise a great number of individual compounds. More than 90 alkaloids have been isolated, for example, from extracts of *Catharanthus roseus* [7]. A lot of them have similar physico chemical properties and chromatographic behaviour. Generally, the alkaloids of *Catharanthus roseus* can be classified into two main groups: monomeric and bis (or binary) indole alkaloids [7, 8]. Catharanthine and vindoline belong to the monomeric group. They are distinguished from the corresponding bis indole constituents for their relatively low polarity.

Precoated glass Merck TLC Silica gel 60 F_{254} 20 cm x 20 cm were preferred in this study due to our experience and conviction in their high chromatographic quality and reproducible results (see also [9]). It is worth noticing that small differences were observed between separate batches but they had negligible effect on the final results. As a matter of fact excellent results have been obtained on aluminium oxide 60 F_{254} type E (Merck, Art. 5715) plates as well.

Silica gel plates were, however, preferred because of their convenience and wider use in practice.

The composition of the mobile phase was established empirically. The chromatographic behaviour of catharanthine and vindoline as well as of bis indole alkaloids vinblastine and leurosine against each constituent of the proposed solvent mixture has been studied. With pure ethyl ether a tailing of some alkaloid spots was observed, the spots being spread. Acetone and ethanol did not cause tailing but was not selective as vindorosine could not be resolved from vindoline, the former migrating just ahead. Petroleum ether alone could not move the components from the origine and was, therefore suitable modifier. Thus, a mobile phase of petroleum ether/ethyl ether/ acetone/ ethanol in proportions 70:10:20:1 (v/v/v/v) was used and it provided reliable chromatographic result: well shaped round spots, clear separation of catharanthine and vindoline from the accompanying components, reasonable distances between the spots satisfying the densitometric requirements. Moreover, under these the bis indole alkaloids remained at the start (or close to it), while catharanthine and vindoline were the main spots with R_f values 0.39 and 0.20 respectively. A lot of non polar minor components, which formed series of well defined spots, were spaced between catharanthine and vindoline but were clearly separated and did not interfere with the quantitative densitometric measurements.

Densitometric quantitation

As already shown [6] the indole alkaloids have strong UV chromofors and can be quantified by measuring the absorbance of the spots directly on the plate via scanning densitometry. In order to determine the optimal wavelength, the respective in situ UV spectra were recorded. Catharanthine and vindoline had maxima at different wavelengths: 280 nm (catharantine) and 310 nm (vindoline) and these were chosen for the quantitative measurements. Thus, the

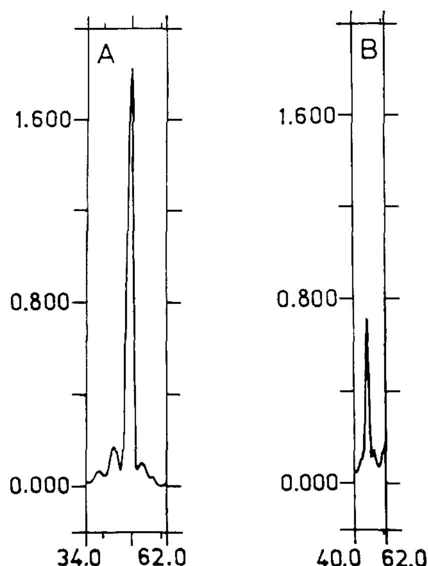

FIGURE 2. Densitograms of Catharntine (A) and vindoline (B) in extracts of *Catharanthus roseus* applaied on silica gel G plates and developed with mobile phase petroleum ether / ethyl ether / acetone / ethanol (70+10+20+1, v/v/v/v).

respective spots were measured separately, each at its characteristic wavelength the scanned distance being about 20-25 mm, Figure 2.

Plates were scanned not longer than 45 min after development. This time was enough for the mobile phase solvents to evaporate while the densitometric signals did not show any significant decrease or increase of the recorded values.

Calibration graphs were constructed by using series of standard solutions of each alkaloid (see EXPERIMENTAL). Standards were spotted in triplicate and peak areas (as derived from the integrator) were plotted against the respective amounts per spot. Both graphs were linear in the interval 1 μg -5 μg per spot, Figure 3. The correlation coefficients were 0.998 (catharantine) and 0.995 (vindoline).

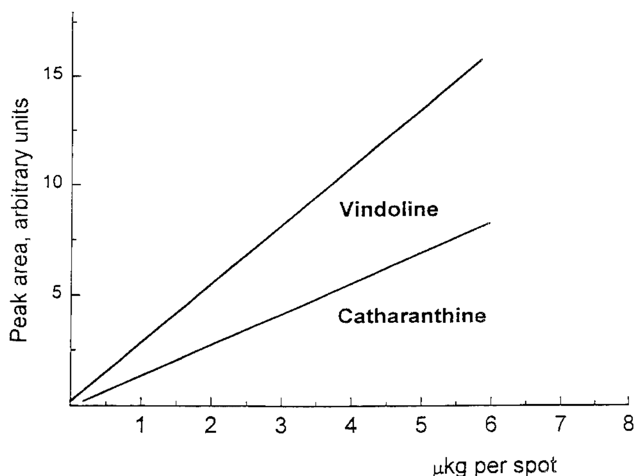

FIGURE 3. The peak area *vs* per spot amounts of catharantine (λ=280 nm and vindoline (λ = 310 nm) as measured by scanning densitometry

Table 1

Acuracy and Precision of the Densitometric Determination of Catharnthine and Windoline by Silica gel Thin Layer Chromatography

Alkaloid	Known, μg/per spot	Found, μg /per spot[a]	Relative error, %[b]
Catharanthine	1.03	1.00±0.03	2.9
	3.09	3.06±0.03	0.9
	5.15	5.05±0.10	1.9
Vindoline	1.03	1.02±0.01	0.9
	3.07	3.08±0.01	0.3
	5.12	5.13±0.01	0.2

[a] mean ± standard deviation, N=2.
[b] $|\mathbf{x}| - |\mathbf{a}|$ **/ a * 100**, where **x** is the mean value and **a** is the known value.

In order to determine the accuracy and the precision of the densitometric measurements catharantine and vindoline were spotted in three different per spots amounts on two different plates. The respective standards, in concentrations of 2.0 μg/per spot and 4.0 μg/per spot were also applied alongside each plate. Plates were developed as described above and scanned in the automatic external standard mode. The results (in μg per spot as derived from the integrator) are shown in Table 1. It is evident that the standard deviation did not exceed 0.1 μg/per spot and the relative error was not higher than 3%.

This approach has been in use in our laboratory for more than two years and is suitable, in our opinion, for phytochemical screening of plant extracts since it answers all requirements for a reliable analytical method.

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DETERMINATION OF MOLECULAR SPECIES OF TRIACYLGLYCEROLS FROM HIGHLY UNSATURATED PLANT OILS BY SUCCESSIVE APPLICATION OF SILVER ION AND REVERSED PHASE TLC

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ABSTRACT

A method for the quantitative determination of molecular species of triacylglycerols in highly unsaturated plant oils by consecutive use of different TLC techniques is described. Silver ion TLC in both analytical and preparative mode has been followed by reversed phase TLC to give results compatible with those obtained with RP-HPLC and capillary GLC. The method has been applied to corn and cotton seed oils. The number of the separated and quantified triacylglycerol species in these oils prevails those reported in the literature.

INTRODUCTION

The detailed determination of the triacylglycerol (TAG) composition of natural fats and oils has always been one of the most important but difficult tasks in the lipid analysis. The analyte is a complex mixture of molecular species with very similar chemical properties and chromatographic separation is absolutely necessary in

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order to obtain reasonable results. It is accepted now that there is no single chromatographic method capable to provide complete resolution of all components of a natural TAG mixture. A properly chosen sequence of chromatographic separations provides much more detailed and unambiguous information on sample composition [1] than does any single method irrespectively of the principles and instrumentation used. Among the chromatographic techniques available, silver ion chromatography has a key position in that it separates triglycerides on the basis of a single molecule property - degree of unsaturation [2]. Subsequent fractionation by high temperature gas liquid chromatography (GLC) reversed-phase thin-layer chromatography (TLC) or reversed-phase high performance liquid chromatography (RP-HPLC) is based on the different chain-length or overall polarity of the TAG molecules.

The great value of the complementary separations of TAG by silver ion chromatography and reversed phase chromatography was recognised long ago and the achievements has been recently reviewed [3]. Naturally, in the early seventies most attention was paid to the combination of silver ion TLC (Ag-TLC) and reversed-phase TLC (RP-TLC). Unfortunately, in those days both Ag-TLC and RP-TLC were messy techniques that were not easy to control. The results were mainly qualitative [2]. *In situ* quantitation was examined [4] but found no wide application and messy procedures which included scrapping, elution and transmethylation of the zones, and GC analysis of the component fatty acids were usually applied. Most of the drawbacks of both Ag-TLC and RP-TLC were, however, successfully overcomed and at present a well established procedures which include *in situ* quantitation by densitometry are available [4-9]. On the other hand, for a long period, RP-HPLC was considered as the only technique capable to solve all problems of the TAG analysis. However identification of dienoic and trienoic plant TAG was and definitely is neither easy nor complete and detection problems hampered the quantitative analysis in great extent. Thus, the sequential application of Ag-TLC and RP-TLC, provided the most detailed quantitative information on the TAG structure of sunflower oil, olive oil and peanut oil [7,9] before the successful utilization of a stable silver ion column by Christie [10] converted the powerful combination of Ag-HPLC and RP-HPLC into a handy and convenient analytical procedure [11,12,13]. However, a limited number of natural TAG mixtures has been analysed by the complementary application of these two methods at present and most of the published results relayed on RP-HPLC only.

Obviously, TLC techniques can not compete in speed with the HPLC, but are efficient alternatives to the more expensive and sophisticated HPLC procedures. In the preparative mode presented bellow Ag-TLC can be a successful aid to subsequent RP-HPLC. Moreover, it is demonstrated in this work that the combination of silver ion TLC and reverse phase TLC is capable to resolve complex mixtures of unsaturated seed oils TAG such as corn and cotton. The number of the separated and quantified TAGs species in these oils prevails those reported in the literature.

EXPERIMENTAL

Materials, Chemicals and Samples

All reagents and solvents were analytical grade. Solvents were distilled before use. Petroleum ether was a b.p. 40-60°C fraction. Diethyl ether was peroxide-free, and chloroform, when used as a mobile phase component, was treated to remove the stabilizing alcohol. Dimethyldichlorosilane (DMDS) was purchased from Fluka (Switzerland) and was used as a silanizing reagent. Kieselguhr G and silica gel G were obtained from Merck (Germany). Sulfuryl chloride (Merck, Germany) and 50% solution of sulfuric acid in ethanol were used as charring reagents.

Corn and cotton oils were purchased from local suppliers.

TAG fraction was isolated by preparative silica gel TLC (1 mm thick layer) with a mobile phase of petroleum ether - acetone, 100:10 (v/v). The purified TAG were dissolved in hexane to give a 0.5% solution.

A standard mixture was prepared by mixing equal quantities of purified TAGs from lard and sunflower oils purified as described above; added to this mixture was certain amount of tristearine in order to increase the proportion of the trisaturated TAG (SSS, S, for saturated fatty acid moiety) to a reasonable value. This mixture was used to identify the TAGs from SSS to DDD (D-dienoic fatty acid moieties). A pure TAG fraction from tangerine oil with known composition [14,15] was used to identify TAGs which contained linolenic acid (trienoic fatty acid or T).

Ag-TLC

Quantitative mode

The procedure is described in details elsewhere [5,14,15]. Briefly, TAG classes differing in unsaturation were separated on 19 x 4 cm glass plates, coated

with ca 0.2 mm silica gel G layer and impregnated by dipping with a 0.5% methanolic solution of silver nitrate. An aliquot of 5-10 μl of the sample (about 20-40 μg) was applied to a plate. Plate was developed with a defined volume of the mobile phase in open cylindrical tanks (24 cm x 5 cm i.d) and the whole volume was allowed to pass through the plate. It was then dried (1 hour at 110°C), and treated consecutively with bromine and sulphuryl chloride vapours (30 min each, in closed tanks and in fume-cupboard). The separated TAG classes were finally charred by heating at 180-200°C on temperature-controlled metal plate.

Preparative mode

Preparative Ag-TLC was carried out as described in [6]. Namely, TAG classes were separated on 20x20 cm home-made glass plates covered with ca. 1 mm thick silica gel G layer which contained 5% silver nitrate. Plates were sprayed with 2',7'-dichlorofluorescein and TAG zones were vizualised under UV light. They were scrapped, transferred to small chromatographic columns and eluted with diethyl ether. The purity and identity of each zone was checked by analytical Ag-TLC after cochromatographing with the reference TAG mixture and the source oil, applied alongside. The solvent was removed by evaporation under nitrogen and samples were redissolved in hexane to give a 0.1% solution.

Quantitative RP-TLC

The procedure described in reference [9] was applied. In brief, 19 x 4 cm glass plates covered with ca. 0.2 mm thick Kieselguhr G layer were first treated for 6 hours with vapours of DMDS and then washed by a single elution with methanol. A 5-10 μl aliquot of the 0.1% TAGs chloroform solution was applied on the plate and developed twice in closed cylindrical tank (dimensions as shown above), each time with fresh 3 ml of the mobile phase to a solvent front of 17 cm. A mixture of acetone/acetonitrile/water was used as a mobile phase. The ratio acetone/acetonitrile was kept constant, 7/3 (v/v), while the proportion of water was varied depending on the TAG composition.

Plates were dried (at 110°C for 1 hour) and separated TAG species were vizualized by spraying with 50% ethanolic sulphuric acid and heating at 200-220°C for about 5 min over a temperature-controlled metal plate.

Densitometry.

The densities of the charred spots were measured by a Shimadzu CS-930 densitometer, equipped with DR-2 Shimadzu integrator, in the zigzag reflection mode at 450 nm. Beam-slit was varied from 0.4x0.4 mm to 1.2x1.2 mm depending on the separation achieved. The quantity of each spot was presented as relative area percent, as derived from the integrator.

Two sets of densitometric results were obtained: Ag-TLC provided the quantitative data for the TAG classes differing in unsaturation and RP-TLC - for the TAG species differing in chain-length within a given class. Obviously, the Ag-TLC results were of vital importance as they were used as a base to recalculate the RP-TLC results and to produce the final data for the TAG composition of the sample.

RESULTS AND DISCUSSION

Ag-TLC

Cotton and corn oil are of certain industrial interest and have been intensively studied by Ag-TLC [16,17,18,19] and RP-HPLC [20,21]. Therefore, they were used in this study to demonstrate the ability of the combination of Ag-TLC and RP-TLC in TAG analysis, as it was possible to compare our results with those obtained by others.

Our experience in Ag-TLC revealed that three different developments on three different plates are necessary in case linolenic acid is present in the sample, even at contents lower than 1% [14,22]. The chromatographic conditions are presented in Table 1.

The first column presents the condition suitable for separation and determination of the SSS, SSM (M, for monoenoic fatty acid moiety) and SMM classes (Fig.1-A). SSS was not found in the examined oils even by heavy overloading. The SMM/SSM ratio was determined under this conditions.

On a separate plate, under the conditions presented in the second column we were able to separate all TAGs but SSM (Fig. 1B). These conditions did not provide satisfactory resolution of TAG classes with higher unsaturation than SDD. These TAG classes (denoted further in the text as "SPUTAG" (Sum of the Polyunsaturated TAG)) therefore were quantified as a sum. The critical point at

TABLE 1.

Chromatographic Conditions for the Separation of TAG Classes by Silver Ion TLC*

	Separation of S_3, SM_2 and S_2M			Separation of TAG from S_2M to T_3			Separation of the poly-unsaturated TAGs (Σp.u.)		
Oil	sample (μg)	mobile phase (v/v)	volume (ml)	sample (μg)	mobile phase (v/v/v)	volume (ml)	sample (μg)	mobile phase (v/v/v)	volume (ml)
cottonseed	35	PE:A 100:5 Fig.1A	7	30	PE:A:EA 100:4:2 Fig.1B	12	20	PE:A:EA 100:4:2 Fig.1C	12
corn	35-40	PE:A 100:5	7	30-35	PE:A 100:8 + 100:5	5 6	25-30	PE:A 100:8 + 100:5	5 6

* PE - petroleum ether (b.p. 40-60°C)
A - acetone
EA - ethylacetate
S - saturated, M - monoenoic, D - dienoic and T - trienoic fatty acid moieties

this stage was the sample size. Overload in densitometry leads to systematically lower results for the overloaded components. We used the ratio SPUTAG/MMD as a criterion to keep overload under control. The sample size which ensured maximal value of the ratio was considered suitable. Quantitation was considered correct in these cases only when SPUTAG/MMD remained constant.

A third plate was used to resolve the components of SPUTAG (Table 1, third column, Fig. 1C). The resolution was complete and enabled correct densitometric determination.

The mobile phase we usually use in Ag-TLC is binary with light petroleum and acetone being mixed in different proportions [5,14,15,22]. This mixture was suitable for corn but not for cotton TAGs. The specific TAG composition of cotton oil required a third component. Ethyl acetate was found suitable as it ensured the separation of TAGs which normally formed critical pair.

RP-TLC

Table 2 presents the chromatographic conditions used to resolve the TAG species within a TAG class by RP-TLC. Obviously, since the oils contained only one monoenoic - oleic, one dienoic - linoleic, and one trienoic fatty acid - linolenic, TAG chain length was determined by the chain-length of the saturated fatty acids.

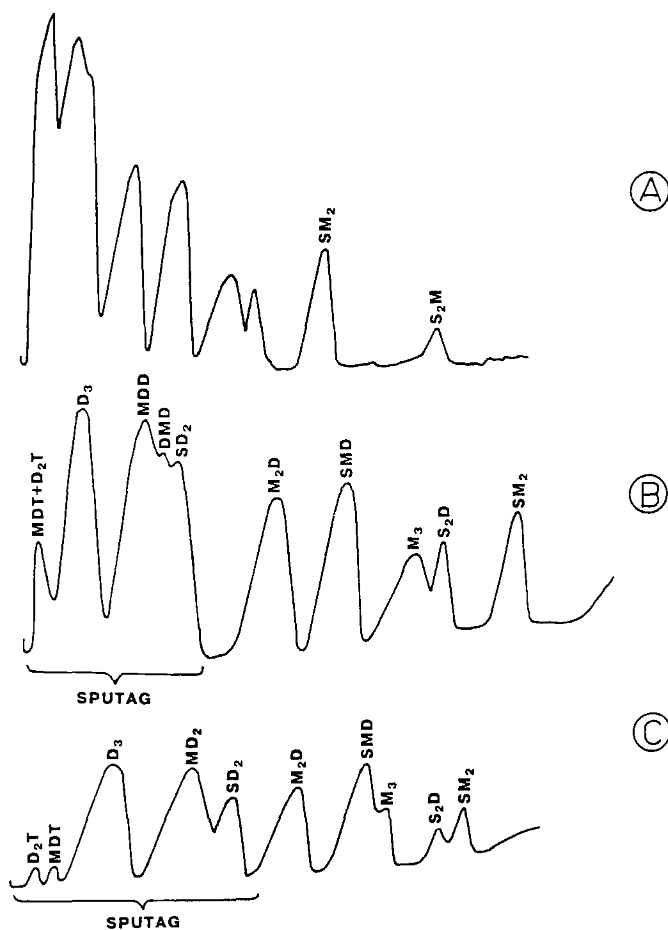

FIGURE 1

Typical resolution of the TG groups of cottonseed oil. For the experimental conditions see Table 1.

TABLE 2.

Water Proportion in the Mobile Phase acetone/acetonitrile/water, 70:30:X for the Separation of Triacylglycerol Classes into Molecular Species by RP-TLC

TG class[a]	TG species[b]	PN[c]	water proportion, by volume
S_2M	PPO, PStO, StStO	48, 50, 52	12
SM_2	POO, StOO	48, 50	14
S_2D	PPL, PStL, StStL	46, 48, 50	12
SMD	POL, StOL	46, 48	18
SD_2	PLL, StLL	44, 46	20

[a] For the abreviations see the footnote to Table 1
[b] The order of designation does not indicate positional isomers, P - palmitic; St - stearic; O - oleic; L - linoleic fatty acid moieties
[c] Partition number PN=C-2n; C - number of carbon atoms
n - number of double bonds

A three component mobile phase was used. Acetone/acetonitrile ratio was kept constant, 7:3 (v/v). Water was suitable modifier [6] and its proportion in the mobile phase was gradually increased with increasing overall unsaturation of the TAG class. In a previous study a simple approach to predict the water proportion necessary for a good separation was established [6].

Quantitation.

It is a well known that while the fatty acid composition of a given seed oil can vary depending on the climate, genetic or variety factors [23,3], the TAG composition varies little, with the proportion between the different unsaturation classes remaining roughly constant and unique [24].

The TAG compositions of cotton and corn oils, as being determined in this study, are presented in Table 3. As already noticed, (see Experimental) resolution and quantitative results obtained by Ag-TLC were the core of the whole analysis. The quantitative result for a given TAG class was taken to recalculate the data for the component molecular species obtained by RP-TLC. Measures were taken to ensure the necessary accuracy and precision of the analysis (see [8] where this problem was studied in details).

TABLE 3.

TG Composition of Cotton and Corn Oils as Determined by Successive Application of Ag-TLC and RP-TLC*

TG classes (number of double bonds)	TG MOLECULAR SPECIES				COTTON				CORN			
					Ag-TLC + RP-TLC	CGC	RP-HPLC	RP-HPLC	Ag-TLC + RP-TLC	CGC	RP-HPLC	RP-HPLC
						ref.20	ref.20	ref.21		ref.20	ref.20	ref.21
S_3 (0)	PPP	16:0	16:0	16:0	-	0.5	-	0.4	-	-	-	-
	PPS	16:0	16:0	18:0	-	0.1	-	-	-	1.9	0.4	-
S_2M (1)	MiPO	14:0	16:0	18:1	**0.4**	0.6	-	-	-	-	-	-
	PPO	16:0	16:0	18:1	**2.3**	6.2	1.5	4.4	**0.6**	3.9	1.5	-
	PStO	16:0	18:0	18:1	**0.9**	1.2	0.5	0.3	**0.3**	0.5	0.1	-
	StStO	18:0	18:0	18:1	**tr.**	0.2	-	-	**0.2**	0.3	-	-
	StAO	18:0	20:0	18:1	-	-	-	-	**0.1**	-	-	-
SM_2 (2)	POO	16:0	18:1	18:1	**2**	4.4	2	2.4	**3.5**	4	2.5	2.2
	StOO	18:0	18:1	18:1	**1.4**	0.6	0.7	0.2	**0.6**	0.7	0.3	-
	AOO	20:0	18:1	18:1	**1.4**	-	-	-	**0.2**	-	-	-
S_2D (2)	MiPL	14:0	16:0	18:2	**0.9**	1.7	0.6	-	-	-	-	-
	PPL	16:0	16:0	18:2	**11.8**	8.1	7.7	15.8	**1.3**	-	-	2
	PStL	16:0	18:0	18:2	**1.2**	2.3	1.1	-	**0.8**	1.9	2.8	1.7
	PAL	16:0	20:0	18:2	-	-	-	-	**0.5**	-	-	-
M_3 (3)	OOO	18:1	18:1	18:1	**0.7**	2.6	1.6	1.4	**5.5**	4.1	3.1	3.7
SMD (3)	POL	16:0	18:1	18:2	**13.4**	10.4	10.9	12.8	**10.7**	13.8	10.4	9.9
	StOL	18:0	18:1	18:2	**2**	1.3	0.9	1	**1.9**	1.7	1.2	1.5
	AOL	20:0	18:1	18:2	-	-	-	-	**0.7**	-	-	-
M_2D (4)	OOL	18:1	18:1	18:2	**5.5**	6.1	5.1	4.7	**13.7**	11.2	10.9	10.4
SD_2 (4)	MiLL	14:0	18:2	18:2	-	1.1	-	-	-	-	-	-
	PLL	16:0	18:2	18:2	**20.5**	20.8	24.5	25.4	**8.4**	17.1	16.4	15.2
	StLL	18:0	18:2	18:2	**4.5**	1.7	1.1	1.8	**1.7**	2.1	1.9	1.8
	ALL	20:0	18:2	18:2	-	-	-	-	**0.2**	-	-	-
S_2T (3)	PPLn	16:0	16:0	18:3	-	-	1.4	-	-	-	0.1	-
MD_2 (5)	PoLL	16:1	18:2	18:2	**}14.4**	0.1	0.3	-	**}27.1**	-	-	-
	OLL	18:1	18:2	18:2		14.1	15.4	11.8		18.8	22.5	26.1
M_2T (5)	OOLn	18:1	18:1	18:3	**tr.**	-	-	-	-	-	0.3	-
D_3 (6)	LLL	18:2	18:2	18:2	**17.4**	15.9	23.2	15.6	**19.2**	17.7	25.6	25.2
MDT (6)	OLLn	18:1	18:2	18:3	**0.3**	-	-	-	**1.7**	-	0.6	-
D_2T (7)	LLLn	18:2	18:2	18:3	**0.2**	-	1.5	-	**1.2**	0.3	1.2	2.1

* For the abreviations see the footnotes to Table 1 and 2; Mi and 14:0 - myristic acid; 16:0 - palmitic acid; 18:0 - stearic acid; A and 20:0 - arachidic acid; B and 22:0 - behenic acid; Po and 16:1 - palmitoleic acid; 18:1 - oleic acid; 18:2 - linoleic acid; 18:3 - linolenic acid moieties.

Results obtained in this work were compared with those reported by others where chromatographic methods, like gradient RP-HPLC and capillary GLC [20,21] were employed. Between the numerous papers only these were chosen where the TAG composition has been determined experimentally and not by calculations [25].

Cotton oil. This oil is widely used for nutrition purposes either alone or in mixtures with soybean oil [26] and was, therefore, intensively studied and analyzed [16-21]. It has a relatively high content of saturated fatty acids (25-30%) which is not typical for the most abundant seed oils. In total, 19 molecular species have been determined in this study. Minor components such as MiPO, PStO, MiPL, OOO, OLLn, LLLn, and MiPO have been determined. Of these, MiPO has been determined with capillary GLC only [20] and OLLn has not been found at all. Our results are in a good general agreement with those works where Ag-TLC followed by GLC have been applied [16-19].

Corn oil. A specific feature of this oil is the relatively high content of the symmetric positionally isomeric DMD TAG as found by enzyme hydrolysis [17]. It has been found that the high DMD content hampers the clear resolution of the SD_2/MD_2 classes by Ag-TLC [22,27]. To avoid the partial resolution of the positionally isomeric TAG, the plate was given two successive developments; the second mobile phase being of lower polarity (Table 1, columns two and three).

There is a good agreement between our quantitative Ag-TLC results and those reported previously, with one exception: the SD_2 and MD_2 classes. While the sum of these TAGs coincide very well with the published results, we found a higher quantity of MD_2 and lower for SD_2 then the reported. The reason might be that in [16] and [17] a combination of preparative Ag-TLC with GLC was used. Under the reported conditions of preparative Ag-TLC both TAGs migrate as two, not clearly resolved, zones and were isolated together. The high content of silver nitrate in the layer (13%) usually hampers strongly the detection under UV. Presumably, the zones haven't been precisely located and differentiated.

We determined 22 TAG species in corn oil and this number is higher than has been achieved by any chromatographic method so far. Recent communications employing RP-HPLC reported 19 [20] and 12 [21] species (Table 3). In some extent, the differences are due to the presence of low percents of stearic and

arichidic acids in corn oil. Exactly the minor TAG components containing these acids, were not resolved and determined by RP-HPLC. This is an obvious result since the total sample was injected onto the column, mixed peaks were inevitably formed and the minor component were presumably lost.

On the other hand, while the sum of PLL and OLL found in this work equals that in [20], we determined a much lower PLL content (Table 3). Under the conditions of [20], PLL and OLL differ by 0.51 ECN (0.64 TCN). These values seem to be not a sufficient difference for a base-line resolution of the two neighboring TAG. In the sequence of methods used in the present work PLL and OLL appeared in different unsaturation fractions and were quantified separately. We assume, therefore, that in the present work their proportion had been correctly determined.

Based on the TAG composition of the samples, their fatty acid compositions were calculated. The values obtained are compared with those determined directly by GLC in Table 4. There is a very good agreement especially if one takes into account that 19, respectively 22, TAG species were used to calculate the fatty acid composition. This is an evidence for the high accuracy of the analysis.

TABLE 4.

Comparison of the Calculated from TAG Data Fatty Acid Compositions of Cottonseed and Corn Oils with those Determined Directly by GLC *

Fatty acid composition	Cottonseed oil		Corn oil	
	GC	calcul. from TG	GC	calcul. from TG
14:0	0.9	0.4	<0.1	-
16:0	22.9	22.5	10.8	9.9
16:1	0.5	-	<0.1	-
18:0	1.9	3.3	1.5	1.9
18:1	16.8	17.9	29.0	31.3
18:2	56.4	55.5	57.5	55.4
18:3	0.3	0.2	0.6	0.9
20:0	0.2	-	0.2	0.5
20:1	-	-	0.1	-
22:0	0.1	-	<0.1	-

* Saturated fatty acids under 0.2% and monoenoic fatty acids under 1.0% could not be determined by the RP-TLC procedure used.

We assume that the results reported here are an illustration that the detailed TAG analysis requires preliminary fractionation by silver ion chromatography. Moreover, since it determines the accuracy of the final results, it is of vital importance for this resolution to be as complete and precize as possible. The second chromatographic stage in the analysis, irrespective of the technique applied (RP-TLC, GLC, RP-HPLC), uses then samples with simpler composition. Minor components could be unambiguously resolved, identified and determined as their proportions in the fraction are favourably changed.

We are convinced that the results presented here clearly show that despite its simplicity, TLC is able to provide results which are comparable and even superior to those obtained so far by HPLC.

We also believe that TLC has a certain advantage: the chromatogram on the plate, like a photograph, presents the real state of resolution under the chosen experimental conditions which helps a lot to change them toward the desirable direction. Moreover, identification of the components is much easier and unambiguous since a cochromatography with standards of known composition is always possible.

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QUANTITATIVE ANALYSIS OF ALACHLOR AND ATRAZINE IN POLYMERIC MICROCAPSULES DETERMINED BY REVERSE-PHASE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY WITH DENSITOMETRY

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ABSTRACT

A method to analyze polymeric microcapsules of the herbicides alachlor and atrazine by reverse-phase high performance thin layer chromatography (RP-HPTLC) has been developed. The herbicidal concentration is determined by densitometry. The method is rapid and reproducible and offers a practical alternative to determination of alachlor and atrazine by elemental analyses. Impurities, metabolites, and decomposition products which may yield falsely high percentages of herbicidal content as determined by elemental analyses are readily detected by RP-HPTLC and are not sources of error.

INTRODUCTION

Recently, concern over the pesticide contamination of groundwater has mounted. Selected pesticides have been detected at extremely low levels in groundwater in isolated

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locations across the United States. In 1986, the U. S. Environmental Protection Agency disclosed that at least 17 pesticides used in agriculture had been found in groundwater in 23 states (1). According to a 1988 interim report, 74 different pesticides have been detected in the groundwater of 38 states from all sources. Contamination attributable to normal agricultural use has been confirmed for 46 different pesticides detected in 26 states (2).

The chief objectives of our research are to develop pesticide formulations that will maintain or increase efficacy against target organisms and that will not adversely impact on the groundwater. Microencapsulation is one method for obtaining this goal (3, 4). Microencapsulated pesticides should be safer to handle, exhibit controlled-release properties (thus possibly reducing the total amount of pesticide used), and have reduced potential for leaching in the soil profile while maintaining effective biological activity.

The herbicides alachlor and atrazine frequently have been implicated in groundwater contamination (1, 2). Previously, we reported the preparation of polymeric microcapsules of atrazine and the evaluation of their efficacy as herbicides under greenhouse conditions (5). Polymeric microcapsules of alachlor have also been prepared and evaluated in the greenhouse (O. D. Dailey, Jr. and C. C. Dowler, unpublished results).

In our continuing evaluation of polymeric microcapsules of atrazine and alachlor for long-term stability, volatility, and leaching properties, we sought an inexpensive, rapid, accurate, and reproducible method for determination of herbicidal content. In the past, herbicidal content was determined by the relatively expensive elemental analysis performed by a commercial laboratory. Often the percentage active ingredient determined

from N microanalysis did not agree with that obtained from Cl microanalysis, and resubmittal of samples was necessary. We have investigated high performance thin layer chromatography (HPTLC) with densitometry (6-8) to determine herbicidal content of polymeric microcapsules of alachlor and atrazine. HPTLC has been used for the quantitfication of atrazine and simazine in water (7) and reverse-phase HPTLC (RP-HPTLC) has been used as a simple and direct method of analysis of soils for atrazine and its metabolites (8).

MATERIALS AND METHODS

Chemicals and Reagents.

Technical atrazine [mp 175-177 °C; lit mp: 176 °C (9)] was provided by CIBA, Greensboro, North Carolina. Technical alachlor (provided by Monsanto, St. Louis, Missouri) was recrystallized from 95% ethanol affording material of mp 39.1-41.9 °C (lit mp: 39.5-41.5 °C) (9). Samples of the 88% hydrolyzed polyvinyl alcohols Airvol 205 (low viscosity) and Airvol 523 (medium viscosity) were provided by Air Products and Chemicals, Inc., Allentown, Pennsylvania. Stock 0.5% solutions of Airvol 205 and 523 were prepared by adding the polyvinyl alcohol to the vortex of stirred cold water in a steady stream followed by heating at 85 °C for about 30 minutes. The following polymers were purchased from Aldrich Chemical Company, Inc.: cellulose acetate butyrate, butyryl content 17%, Tm 235 °C (CAB); ethyl cellulose, ethoxyl content 48%, viscosity (5% solution in 80/20 toluene/ethanol) 22 centipoises [EC22]; ethyl cellulose, ethoxyl content 48%, viscosity 100 cps (EC100); poly(methyl methacrylate), low molecular weight (PMML); poly(methyl methacrylate), medium molecular weight

(PMMM). HPLC Reagent grade dichloromethane (DCM) and methanol (MeOH) were used as solvents.

Preparation of Polymeric Microcapsules.

Atrazine and alachlor were microencapsulated within cellulose acetate butyrate, ethyl cellulose of two different viscosities, and low and medium molecular weight poly(methyl methacrylate) by the solvent evaporation process using two different emulsifiers as previously reported (5).

In subsequent discussions, a polymeric microcapsule formulation will be referred to in abbreviated form, such as CAB-205, indicating the use of the polymer cellulose acetate butyrate and the emulsifier Airvol 205.

The herbicidal content of all the polymeric microcapsules prepared was determined by elemental analysis at the time of preparation. Based upon the amounts of materials used, each of the polymeric microcapsule formulations should contain 20% active ingredient. Determination of the herbicidal content of the CAB, EC22, and EC100-523 atrazine formulations was based upon nitrogen and chlorine microanalyses. The atrazine content of the PMML, PMMM, and EC100-205 formulations was determined from nitrogen microanalyses only. High values for the chlorine content of the PMML-523 and PMMM-523 formulations suggested the presence of residual dichloromethane. Chlorine microanalysis for PMML-205, PMMM-205, and EC100-205 were not done due to sample insolubility.

Preparation of Samples for Thin Layer Chromatography.

Standard solutions of alachlor (1.008 μg/μl) and atrazine (1.000 μg/μl) were prepared in methanol. Solutions of

microcapsule formulations were typically prepared by dissolving 250.0 mg of microcapsules in 100 ml of methanol or DCM giving an effective microcapsule concentration of 10.00 µg/4µl. All polymeric formulations dissolved readily in DCM, but only EC formulations dissolved completely in methanol. At least 24 hours were allowed for complete dissolution of alachlor or atrazine from the partially soluble CAB, PMML, and PMMM formulations. Blank solutions were prepared by dissolving 200.0 mg of each of the polymers in 100 ml of DCM. None of the polymers were detectable at the UV wavelengths used.

Thin Layer Chromatography.

TLC was performed on C-18 high performance reverse-phase Uniplates (10 X 20 cm, 150 micron thickness, scored, RP18F; Analtech Inc., Newark, Delaware). Standards and sample extracts or solutions were drawn into microcapillary pipets (1.0, 2.0, and 4.0 µl) and applied with a Nanomat III (Camag, Inc.). The mobile phase for alachlor experiments was MeOH:H_20 (85:15), and for atrazine experiments it was MeOH:H_20 (70:30). Each plate was spotted with 1.0, 2.0, 3.0 and 4.0 µl of the applicable standard solution and with 4.0 µl of each of seven sample solutions in duplicate. In a few instances, the seventh sample was spotted in triplicate. Spotted plates were equilibrated in a development tank containing the mobile phase for 20 min. prior to development. Plates were developed for a distance of 10 cm, dried for at least 15 min., and scanned at 200 nm for alachlor or 220 nm for atrazine with a variable wavelength Shimadzu CS9000U Dual-Wavelength Flying Spot Scanner. Development time was 25 min. for alachlor experiments and 30 min. for atrazine experiments.

Statistical Analysis.

The RP-HPTLC standard curves were analyzed by linear regression analysis (10). The mean alachlor or atrazine concentration in each sample was determined in micrograms and converted to a percentage (based on weight). Variance components were computed using the Maximum Likelihood method available in Proc Varcomp (10) to compare variability among HPTLC runs to variability between duplicate samplings of the same formulation. The t tests (10) were conducted separately on data collected for each formulation, using averages of duplicate samples as a run, to determine whether or not solvent significantly affected the result. Standard errors were calculated for determinations by HPTLC runs, using averages of duplicate samples as a run, and compared to the standard error of % N and % Cl determinations combined, using the homogeneity of variance test (10). The average of HPTLC runs collected for each formulation was paired with the % N (% Cl) result and a paired t-test (10) across all formulations was conducted to determine how closely HPTLC and % N (% Cl) results agreed.

RESULTS AND DISCUSSION

The results of the determination of alachlor content of polymeric microcapsules is given in Table I. The alachlor content in each formulation is given as a percentage (w/w). The percentage alachlor as determined by elemental analysis (based on % N and on % Cl) is given for comparison purposes. All samples listed under the same formulation heading were prepared from the same batch of polymeric microcapsules. Samples denoted by the A, C, and D prefixes were prepared from fresh microcapsules.

TABLE I

Determination of Herbicidal Content of Polymeric Alachlor Microcapsules by RP-HPTLC and Densitometry[1]

Formulation	Sample	Solvent	From RP-HPTLC		From Elemental Analysis		p-value[2]	
			Mean	SE	Mean	Standard Error	Mean	Variance
EC100-205	A1, A2	DCM	17.52[3]	0.39	18.45[4]	2.85	0.5265	0.0173
	C1, D2	MeOH	18.11[3]	0.20	18.45[4]	2.85	------	-----
EC22-205	A3, A4	DCM	17.65[3]	0.32	20.75[4]	2.35	0.0273	0.0170
	C2, D1	MeOH	17.88[3]	0.28	20.75[4]	2.35	------	-----
CAB-205	A5, A6	DCM	18.88[3]	0.32	16.25[4]	6.15	0.3279	0.0000
	D3, D4	MeOH	20.12[3]	0.40	16.25[4]	6.15	------	-----
EC100-205	B1, B2	DCM	16.28[5]	0.24	20.20[4]	1.20	0.0016	0.0700
EC22-205	B3, B4	DCM	16.46[6]	0.37	19.70[4]	2.90	0.0593	0.0118
CAB-523	B5, B6	DCM	19.17[5]	0.31	21.35[4]	0.55	0.0130	0.7099
PMML-523	B7	DCM	19.83[7]	0.45	24.80[4]	0.30	0.0041	0.7206

[1]Alachlor content in each formulation is given as a percentage (w/w). [2]p-Value <0.05 indicates a significant difference between the RP-HPTLC method and elemental analyses. [3]Mean and standard error (SE) based on $n = 8$ runs. [4]Mean and SE based on $n = 2$ runs. [5]Mean and SE based on $n = 6$ runs. [6]Mean and SE based on $n = 7$ runs. [7]Mean and SE based on $n = 3$ runs.

Samples prepared from 4-year old microcapsules are denoted by the B prefix. Duplicate samplings (e. g., A1 and A2 and C1 and D2) were taken from the same batch of microcapsules to test for homogeneity. Samples prepared in dichloromethane (DCM) solution are designated by the prefixes A and B, and preparations in methanol (MeOH) solution are designated by C and D. There were a total of 13 separate HPTLC plate developments or runs. Samples denoted by A were spotted on the same plate, B samples were spotted on the same plate, and C and D samples were spotted together on the same plate. Each individual sample was spotted on at least three different plates. None of the developed TLC plates showed any impurities or decomposition products, indicating long-term stability of the microcapsules.

The following data were obtained from the 13 plate developments. The R-square for the alachlor standard curve varied between 0.990 and 0.999 with a mean of 0.996. The coefficient of variation (%CV) for the duplicate spottings was always under 10%, under 5% ninety-two percent of the time, and under 2% sixty-two percent of the time. These data indicate very high reproducibility in the spotting technique. There was no significant difference in %CV between methanol solutions and DCM solutions, indicating that the higher volatility of DCM was not a source of error in the spotting.

Statistical analysis of the data in Table I leads to the following conclusions. Variability among HPTLC runs is greater than variability between duplicate samplings of the same formulation, as indicated by variance component estimates (4.20 and 0.34 respectively). There is no statistical difference ($p>.19$) between the results obtained with the two solvents, DCM (A samples) and MeOH (C and D samples), as shown by the t-test conducted for

each formulation. The variability among percentage alachlor determinations by HPTLC runs is significantly less than that between the determinations based on % N and on % Cl for CAB-205 only; however, numerically the variability is smaller for the RP-HPTLC method in every case except for PMML-523. Additional elemental analyses would be required to make more thorough variability comparisons. It must be noted that of a total of ten different formulations, the four 4-year old alachlor formulations chosen for examination had the lowest variability between % N and % Cl determination of herbicidal content. Finally, the RP-HPTLC results are generally more in agreement with percentage determination based on % N (p=.9935) than on % Cl (p=.0001), as shown by paired t tests.

The results of the determination of atrazine content of 4.5- year old polymeric microcapsules is given in Table 2. Samples dissolved in methanol are denoted by the prefix E and those dissolved in DCM are denoted by the prefix F. The numerical suffixes denote the same polymeric formulation. The five formulations listed showed no evidence of impurities or decomposition products. Four unlisted formulations (EC22-205, EC22-523, EC100-205, and EC100-523) showed predominantly atrazine (Rf 0.414-0.44), but there was a significant amount of a second compound (Rf 0.501-0.53). The tenth formulation (PMMM-205) showed a compound with Rf 0.475, but no atrazine. Neither of these two unknowns is deethylatrazine, deisopropylatrazine, or hydroxyatrazine, as demonstrated by spotting of authentic samples of these three metabolites and comparison of Rf values (8).

The following data were obtained from the six plate developments employed. The R-square for the atrazine standard

TABLE II

Determination of Herbicidal Content of Polymeric Atrazine Microcapsules by RP-HPTLC and Densitometry[1]

Formulation	Sample	Solvent	From RP-HPTLC		From Elemental Analysis		p-value[2]	
			Mean	SE	Mean	Standard Error	Mean	Variance
CAB-523	E1	MeOH	20.13[3]	0.32	20.40[4]	1.10	0.7901	0.2116
CAB-205	E2	MeOH	19.57[3]	0.19	20.00[4]	1.80	0.7714	0.0311
PMML-205	E6	MeOH	21.13[3]	0.63	20.1[5]	----	0.5003	-----
PMML-523	E7	MeOH	22.53[3]	0.50	19.7[5]	----	0.1045	-----
PMMM-523	E9	MeOH	21.07[3]	0.60	18.8[5]	----	0.0828	-----
CAB-523	F1	DCM	15.57[3]	0.62	20.40[4]	1.10	0.0242	0.5659
CAB-205	F2	DCM	15.07[3]	0.32	20.00[4]	1.80	0.0363	0.0311
PMML-205	F6	DCM	16.23[3]	0.51	20.1[5]	----	0.0227	-----
PMML-523	F7	DCM	17.33[3]	0.18	19.7[5]	----	0.0215	-----
PMMM-523	F9	DCM	15.70[3]	0.50	18.8[5]	----	0.1045	-----

[1]Atrazine content in each formulation is given as a percentage (w/w). [2]p-Value <0.05 indicates a significant difference between the RP-HPTLC method and elemental analyses. [3]Mean and standard error (SE) based on $n = 4$ runs. [4]Mean and SE based on $n = 2$ runs (nitrogen and chlorine microanalyses). [5]Nitrogen microanalysis only.

curve varied between 0.991 and 0.999 for 5 plates and was 0.971 for the sixth. The %CV for the duplicate spottings was always under 10%, under 5% eighty-five percent of the time, and under 2% forty-one percent of the time. These data indicate high reproducibility in the spotting technique.

In contrast to the results obtained for alachlor solutions, the percentage atrazine as determined with DCM solutions of the polymeric microcapsules was uniformly and markedly lower ($p<.0028$) than that determined with methanol solutions. However, the mean percentage of atrazine as determined with methanol solutions was in excellent agreement with the theoretical percentage of 20% and with the percentage derived from % N microanalysis.

CONCLUSIONS

A method employing reverse-phase high performance thin layer chromatography with densitometry in the determination of herbicidal content of alachlor and atrazine polymeric microcapsule formulations has been developed. The method is rapid, accurate, reproducible, and inexpensive (on a per sample basis) and has the added advantage in that impurities, metabolites, and decomposition products (which may yield falsely high percentages of herbicidal content when determined by elemental analyses) are readily detected. In the determination of the herbicidal content of alachlor formulations dissolution of samples in either methanol or dichloromethane gives comparable results. Methanol is the solvent of choice in the analysis of polymeric atrazine formulations. The method should be adaptable to the analysis of formulations of other UV-active herbicides, such as metribuzin and cyanazine.

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TWO-DIMENSIONAL T.L.C. ON MECHANICALLY BLENDED SILICA-BASED BONDED PHASES. EVALUATION OF THE BEHAVIORS OF C8-DIOL MIXTURES USING POLYNUCLEAR AROMATIC HYDROCARBONS AND COMPARISON WITH C18-CYANO MIXTURES

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ABSTRACT

C8-diol mixed phases for two-dimensional T.L.C. were prepared by mechanical mixing of silica-based bonded phases. Their properties in terms of retention and mechanisms developed were determined using polynuclear aromatic hydrocarbons as the compounds analyzed. Comparisons carried out with C18-cyano mixed phases revealed both similar behaviors and appreciably different behaviors, which we have explained.

INTRODUCTION

Two-dimensional thin-layer chromatography (T.L.C.) using two separation mechanisms can be carried out on juxtaposed phases (1).

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When the compounds analyzed are polynuclear aromatic hydrocarbons (P.A.H.), the occupation of the whole layer is difficult. In fact, the retention in the normal mode increases with their resonance energy (2), and similarly in the inverse mode as the hydrophobicity is directly related to the number of fused rings. It was interesting to study whether the mixed phases might be more attractive for the analysis of these environmental pollutants. We have therefore studied some combinations of apolar bonded phases and polar bonded phases, mixed mechanically. The properties of C18/cyano mixed phases have been published recently (3). In this note, we report those of C8/diol mixed phases, not previously studied, and compare them with the results from the earlier work.

MATERIALS

The P.A.H. analyzed were : anthracene (Aldrich) 99.9 % pure, fluoranthene (Aldrich) 98 % pure, benz[a]anthracene (Aldrich) 99 % pure, benzo[b]fluoranthene (Aldrich) 99 % pure, and indeno[1,2,3-c,d]pyrene (Alltech) 98 % pure.

The mechanically mixed C8/diol phases were prepared in various mass compositions from the following two commercial products : Hyperprep 120 octyl (Shandon), d_p (particle diameter) = 12 µm, S (specific surface area) = 200 $m^2.g^{-1}$, % C = 7, bonding ratio = 3.6 $\mu mole.m^{-2}$ and Hyperprep 120 diol (Shandon), d_p = 12 µm, S = 200 $m^2.g^{-1}$, % C = 2,88, bonding ratio = 1.3 $\mu mole.m^{-2}$.

METHODS

Preparation of phase mixtures and plates, and development and detection techniques, were all as previously described (3).

All the results are the mean of three experiments.

RESULTS AND DISCUSSION

Variation of the P.A.H. Retention with the Mass Percentage of the Diol Phase

The R_M = f (mass % of diol phase) graphs given in Figure 1 show the effect of the percentage of the polar phase on the retentions of various P.A.H. in two-dimensional T.L.C. :

- with hexane as solvent in the first direction, the elution mode was normal. In the composition range 30 to 100 % of the diol phase (Fig. 1a), the retention increased linearly with the increase in percentage of the diol phase, whatever the P.A.H. (the correlation coefficients, r, had values : $r_{anthracene}$ = 0.9925 ; $r_{fluoranthene}$ = 0.9875 ; $r_{benzanthracene}$ = 0.9890 ; $r_{benzofluoranthene}$ = 0.9793; $r_{indenopyrene}$ = 0.9937).
- with a 90/10 CH_3OH/H_2O mixture as eluent in the second direction, the mode was of the inverse type. In the composition range 10 to 90 % in diol phase (Fig. 1b), the retention decreased linearly with the increase in percentage of the diol phase, whatever the P.A.H. ($r_{anthracene}$ = - 0.9828 ; $r_{fluoranthene}$ = - 0.9854 ; $r_{benzanthracene}$ = - 0.9934 ; $r_{benzofluoranthene}$ = - 0.9864 ; $r_{indenopyrene}$ = - 0.9846).

Study of the Mechanisms developed by the different C8/diol Mixed Phases according to the Polarity P' of the Mobile Phase.

The graphs in Figure 2 show R_f = f (P'), P' being the polarity as defined by Snyder (4). They correspond to the two

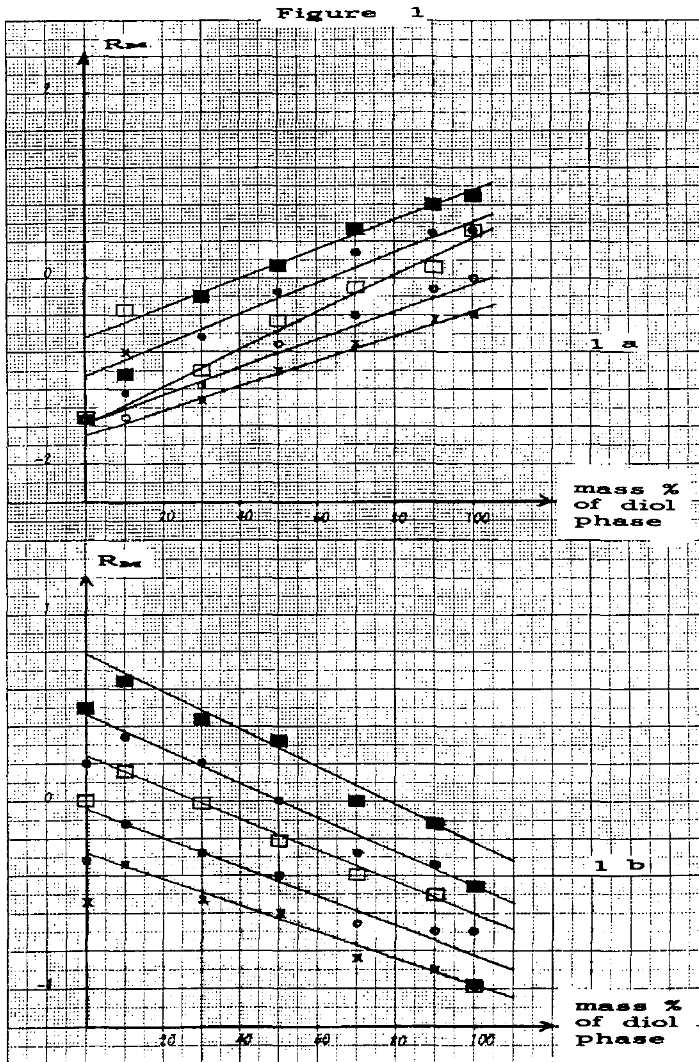

FIGURE 1. Influence of the percentage of the diol phase on the retention in two-dimensional mode.

Compounds : (✕) anthracene, (○) fluoranthene, (□) benz[a]anthracene, (●) benz[b]fluoranthene, (■) indeno [1,2,3-c,d]pyrene. Mobile phases : hexane in the first direction (Fig. 1a) ; CH_3OH/H_2O 90/10 in the second direction (Fig. 1b), after evaporation of hexane.

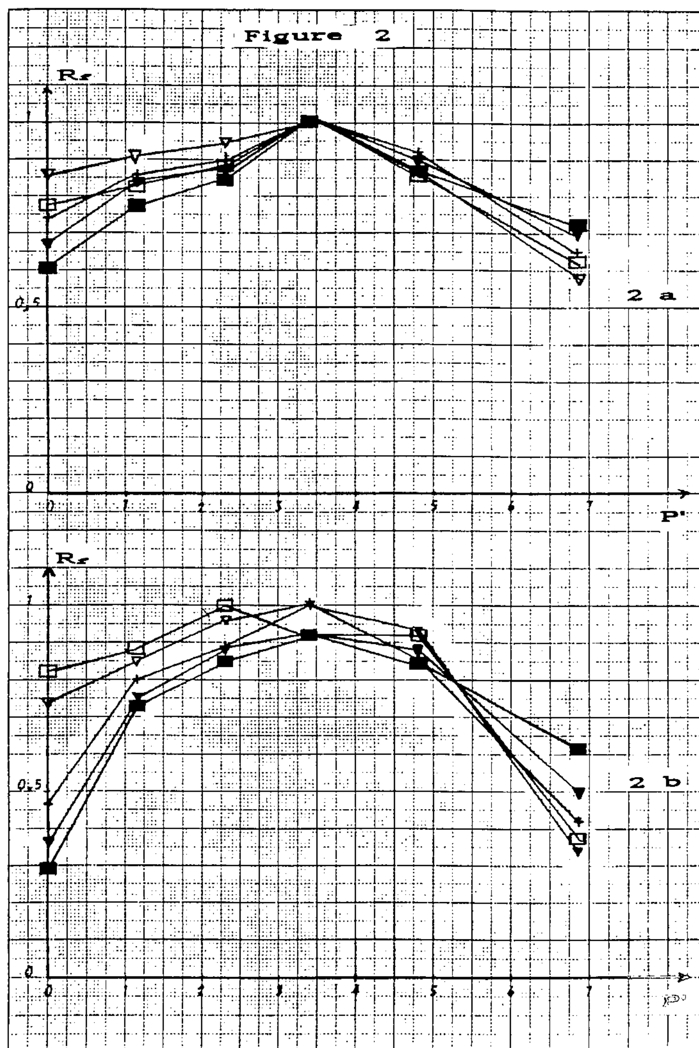

FIGURE 2. Study of the mechanism operating depending on the polarity of the eluent and the composition of the stationary phase.

Compounds : anthracene (Fig. 2a), indeno [1,2,3-c,d]pyrene (Fig. 2b). Mobile phases : P' = 0.0 (hexane), P' = 1.15 (hexane/toluene 50/50), P'= 2.3 (toluene), P' = 3.4 (CH_2Cl_2), P' = 4.8 (dioxane), P' = 6.84 (CH_3OH/H_2O 90/10). Stationary phases : (□) C8, (▽) C8/diol 90/10, (+) C8/diol 50/50, (▼) C8/diol 30/70, (■) diol.

P.A.H. having the greatest differences in retention. The most significant results were those obtained for the most highly retained compound, indeno[1,2,3-c,d]pyrene (Fig. 2b). This was because of a too weak retention of anthracene leading to the R_r observed being too similar as much by modifying P' as the C8/diol mass composition. We observed :

- the existence of a maximum for each curve corresponding to a given mass composition. This maximum, P'_{max}, corresponded to the inversion of the mechanism : normal phase polarity mechanism or inverse phase polarity mechanism according to whether P' was less than or greater than P'_{max} .
- the value of P'_{max} was close to 3.5 for all C8/diol compositions, as well as for the pure diol phase.
- at low polarity, in the range 50-100 % of diol phase, the mixed phases behaved like the diol phase. At high polarity, over the whole composition range, the behavior type was less clear cut, but more resembled that of the C8 phase.

Comparison between the C8/Diol Mixed Phases and the C18/Cyano Mixed Phases

The polarity zones in the mobile phase where the normal phase and the inverse phase mechanisms respectively apply were very comparable. In fact, the whole of the C8/diol mixed phases, and the C18/cyano mixed phases containing at least 50 % of the cyano phase (3), both had a P'_{max} close to 3.5. This was a little unexpected considering the only moderate polarity generally attributed to the cyanopropyl phase.

In normal phase polarities, i.e. P' < 3.5, we will first consider the range 50-100 % in polar phase. On the one hand, the retentions obtained for the P.A.H. on the C8/diol mixed phases were fairly comparable to those obtained on the C18/cyano mixed phases for corresponding mass compositions. On the other hand, the behaviors of these mixed phases were very close to the behaviors of the pure cyano and diol polar phases respectively, themselves also very close. The selectivity triangle for hydrophilic bonded stationary phases (5) shows that the essential property of a cyano phase is the orientation of the dipoles. As for the diol phase, it does not have a dominant acid character, but behaves somewhat oddly as an orienter of the dipoles (5). The retention of the P.A.H., compounds which are not very polar but very polarizable, is essentially governed by Debye-type interactions, which with the cyano and diol phases would lead to similar interaction energies.

In inverse phases polarities, i.e. P' > 3.5, the C8/diol mixed phases showed lower retentions for the P.A.H than those obtained with the C18/cyano mixed phases of corresponding mass compositions. This is explained on the one hand by the higher hydrophobic nature of the C18 group compared to the C8 group (Δlog P = 5.190 determined by Rekker's calculation method (6) in the revised system, P being the partition coefficient in the n-octanol/water system), combined on the other hand with the greater hydrophobic nature of the cyanopropyl group compared to the diol group (Δlog P = 1.181). In addition, for a given polarity of the mobile phase (for example P' = 6.5), the range of retentions

corresponding to the different mass compositions was much narrower for the C8/diol mixed phases than for the C18/cyano mixed phases. This must result from the smaller difference in hydrophobic nature between the individual C8 and diol groups (Δ log P = 5.012) than that between the individual C18 and cyanopropyl groups (Δlog P = 9.021).

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SIMPLE HPLC METHOD FOR THE DETERMINATION OF THYMOQUINONE IN BLACK SEED OIL (*NIGELLA SATIVA LINN*)

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ABSTRACT

A simple and reliable isocratic normal phase HPLC method for the determination of thymoquinone in black seed oil (*Nigella Sativa Linn*, Ranculaceae) is described. After oil extraction with methanol, thymoquinone is analyzed using Econosphere CN column. The mobile phase consists of hexane:2 propanol (99:1 v/v), thymoquinone is monitored by UV detection at 295 nm. This method is quite specific and sufficiently sensitive with a lower limit of 5 nmoles/ml, within day and between-day assays showed variation coefficient below 5%.

INTRODUCTION

Nigella Sativa Linn (Ranculaceae), grows in Mediterranean countries and is cultivated in others. The black seed oil has a long history of folklore medicine in Arabian and other countries for the treatment of various diseases (1,2) such as; asthma, respiratory appression, cough, headache, diuretic, lactagogue and others. The main constituents of black seed oil are fixed oil, volatile oil and alkaloids.

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Investigators attributed the pharmacological activities of black seed oil to its thymoquinone content (the main constituent of the volatile oil) which represents 18.4 - 24% w/w of the volatile oil (3,4). Other constituents detected in black seed oil were thymol, dithymoquinone (thymoquinone dimer), monoterpenes, phenols and some ester (5,6).

El-Tayeb (7) described his own experience in folk medicine and the success he had achieved in treatment of various systemic and dermatological diseases following treatment of patients which extract of the black seed oil or seeds powder either alone or mixed with some other natural products. Abou-Basha et al (8) recently reported a thin layer chromatography quantitative assay of thymoquinone in black seed oil with a limit of detection of 100 nmoles/ml.

Accordingly, we describe here a simple, reliable, more sensitive, and rapid HPLC assay for thymoquinone in black seed oil that could be routinely performed in most laboratories.

MATERIALS AND METHOD

Chemicals

Thymoquinone authentic with 99.9% purity was purchased from Aldrich Chemical Co., Milwaukee, WI, USA. Hexane and 2 propanol were HPLC grade (Springfield, New Jersey, USA). The two black seed oil analysed were obtained from the local market.

Chromatography

The HPLC system consisted of a Bio-Rad 1350 solvent delivery pump, a Rheodyne model 7125 injector, a Waters Lambda Max 481 variable wavelength detector set at 295 nm and a Hewlett-Packard 3394 A integrator. The column used was CN normal phase (250 mm x 4.6 mm I.D., Econosphere™ CN, particle size 10 μ) purchased from Alltech Associates, Inc, Deerfield, IL.,USA.

Sample preparation

1 ml of methanol was added to 1 ml of oil (commercial black seed oil) in a glass centrifuge tube with cover. Vortex mix for 2 mins. The methanol top layer was transferred to a glass

tube. The methanol was evaporated under nitrogen stream. The residue was reconstituted with 1 ml of mobile phase and 20 μl injected into HPLC system.

RESULT AND DISCUSSION

Chromatograms

A typical chromatogram of thymoquinone was presented in Figure 1, Chromatogram of thymoquinone in oil was shown in Figure 2.

Thymoquinone has retention time of R_t 4.48 min at 295 nm. At this wave length there is no interference neither from dithymoquinone (thymoquinone dimer) nor from thymol. Dithymoquinone and thymol were detected at 260 and 275 nm respectively.

Linearity

The calibration curve of thymoquinone were constructed over the range of 0.1-30 nmoles with a correlation coefficient 0.999 (n=6). Each determination (n=6) of the thymoquinone content of black seed oil consisting of calibration curve and oil extracts of interest, were done on the same setting. The lower limit of detection was 5 nmoles/ml.

Variability and percentage recovery

High and low valued quality control sample (30 and 0.5 nmoles) were assayed six times a day on the same day and several days during a two-weeks period, to evaluate the precision of the assay. The within day variability (coefficient of variation) were 2.0 and 3.0 respectively for 30 and 0.5 nmoles. The day to day variation 3.5 for both values. The black seed oil sample 3 (1 ml) spiked with 500 nmol internal thymoquinone standard. The spiked sample extracted as in sample preparation and 20 μl injected into HPLC system. The spiked sample assayed 6 times during two weeks period. The recovery of thymoquinone from spiked sample 3 was 100% with a coefficient of variation 2.5%.

Table 1 summarizes the results for the quantitative assay of thymoquinone as the main active constituent of the volatile oil in black seed oil.

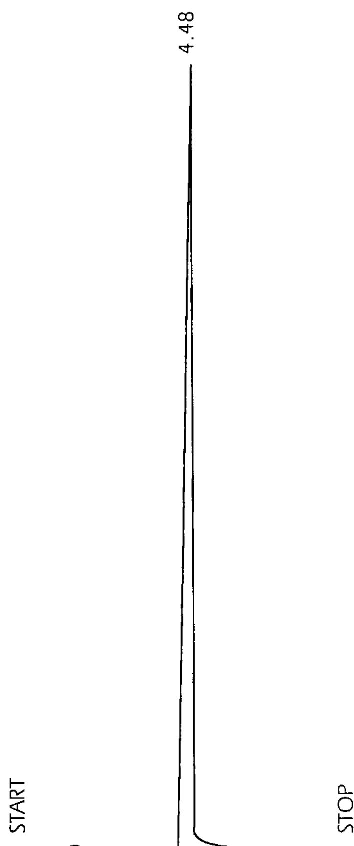

Figure 1. Chromatogram of thymoquinone authentic sample. Column CN (25cm x 4.6mm i.d., Econosphere CN, particle size 10μ); mobile phase: hexane:2-propanol (99:1 v/v); flow rate: 1ml/min; chart speed; 0.5cm/min; temperature: 23° C; detector; UV 295nm; sensitivity: 0.01aufs; sample quantity: 10 nmol.

It has been shown that the volatile oil revealed some pharmacological activities such as bronchodilators (9, 10, 11), increases bile flow and concentration of bile salts (12) and decreases blood pressure in dogs (13) and in rats (14). Indeed, Marozzi *et al* (15) claimed that the pharmacological activities of black seed oil is due to its thymoquinone contents which varies according to the method of manufacturing the oil. This is verified by the results shown

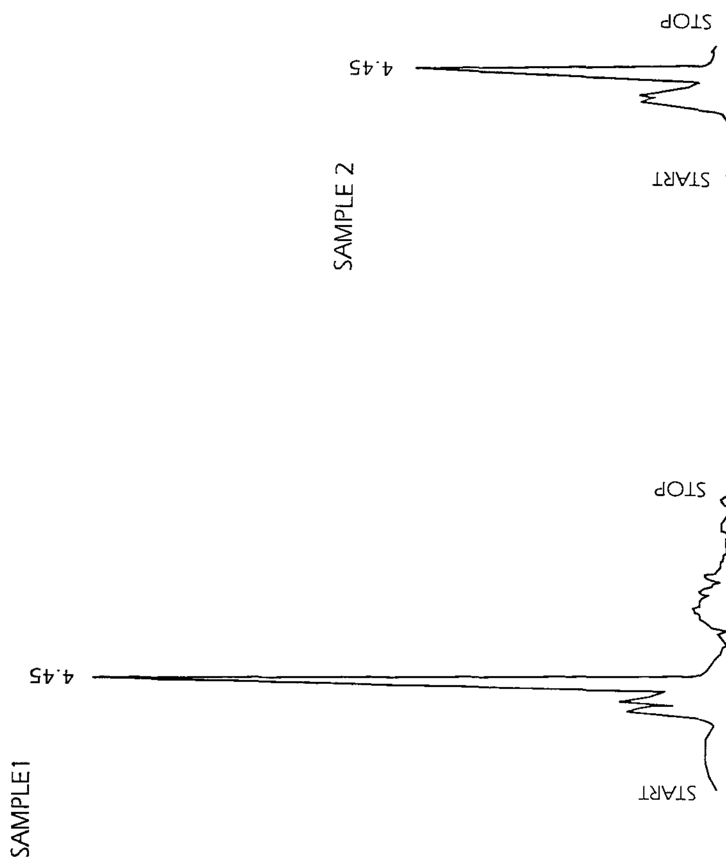

Figure 2. Chromatogram of oil sample 1 and 2. Column CN (25cm x 4.6mm i.d., Econosphere CN, particle sifze 10μ); mobile phase: hexane : 2-propanol (99:1 v/v); flow rate: 1ml/min; chart speed: 0.5cm/min; temperature 23°C; detector UV 295nm; sensitivity: 0.01aufs.

Table 1. Analysis of thymoquinone content in commercial black seed oils and sample 3 spiked with thymoquinone internal standard

Black seed oil	Mean* ± S.D. nmoles/20 μl	CV %	Thymoquinone nmoles/ml
Sample # 1	55.5 ± 1.78	3.2	8.3×10^3
Sample # 2	16.1 ± 0.58	3.6	2.4×10^3
Sample # 3	undetected	-	<5
Sample # 3 spiked	10.0 ± 0.25	2.5	0.1×10^2

* Mean of 6 determinations

in Table 1. Oil sample #1 has a higher thymoquinone content than oil sample #2 while thymoquinone is not detected in oil sample # 3. This requires the drug regulatory authorities to set up a standard limit for this active constituent (thymoquinone) in black seed oil, in order to set up a quality control criteria for this preparation.

CONCLUSION

A simple and reliable method for rapid determination of thymoquinone in black seed oil have been developed.

The HPLC procedure is sensitive with a lower limit of 5 nmoles/ml and a coefficient of variation less than 5% The method is suitable for routine analysis or thymoquinone in black seed oil.

ACKNOWLEDGEMENTS

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ANALYSIS OF BENZOQUINOLINES AND ACRIDINES IN A BRAZILIAN DIESEL OIL BY PARTICLE BEAM LC/MS AND HPLC/UV

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ABSTRACT

Nitrogen bases were isolated from a Brazilian diesel distillate by acid extraction. With minimum sample preparation and clean-up, nitrogen bases were analyzed by particle beam LC/MS and HPLC with photo diode-array detection using reversed phase chromatography. Benzoquinolines were identified as the major nitrogen containing compounds in this basic fraction. By using neutral mobile phases, benzoquinoline homologues were separated, enabling rapid class characterization as well as preparative HPLC isolation of individual benzoquinoline homologues. Acidified mobile phases, however, exhibited greater resolution for individual isomers. UV spectroscopy was used to differentiate various types of benzoquinolines (e.g. benzo[f]quinolines, benzo[h]quinolines, and acridines). Acridines can be easily distinguished from benzo[h]- or benzo[f]quinolines by its different UV absorption spectrum. Benzo[h]- and benzo[f]quinolines were differentiated by the differences in the first derivative of the absorbance ($dA/d\lambda$) vs. wavelength. To confirm the identification, nitrogen bases were also analyzed by conventional GC/MS methods.

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INTRODUCTION

Interest in understanding the organic nitrogen bases that are found in petroleum is associated with several of their undesirable physical and chemical properties[1]. The adverse effects of nitrogen bases in petroleum products are mainly in three areas: a) they poison the catalysts used in the cracking and hydrocracking reforming processes[2, 3], b) they contribute to the instability of fuels during storage[4 - 7], and c) they possess potentially carcinogenic and mutagenic activities[8]. The nitrogen bases found in petroleum generally occur as complex mixtures of alkylazaarenes (nitrogenated polyaromatic hydrocarbons) containing mainly one nitrogen atom[9, 10]. Alkyl-substituted quinolines and benzoquinolines have been reported as major basic nitrogen compounds in crude petroleum[7, 10]. Although much work has been done with regard to the characterization of these compounds in petroleum products, the determination of the precise location of the nitrogen atom in these polyaromatic molecules remains a challenge[10, 11]. It is also of importance to distinguish different classes of azaarenes and to study the distributions of individual isomers because of the information that can be gained relating to the geochemical formation pathways of petroleum[10]. For instance, it is known that the distribution of homologous alkyl-substituted aromatic nitrogen compounds differ widely in their origins and geochemical histories[10]. The toxicity of these nitrogen compounds can also be related to the location of the nitrogen atom[10]. In addition, many of these compounds represent key links in identifying the higher molecular weight nitrogen compounds that are invariably present in petroleum.

Previously, we reported the fractionation and characterization of basic and neutral nitrogen compounds from a Brazilian diesel oil sample[12]. We emphasize in this paper a more complete characterization of the basic nitrogen compounds through an analytical sequence involving complementary chromatographic and spectroscopic techniques. By a combination of LC/MS, GC/MS, and HPLC with online photo diode-array detection, we demonstrate an analytical approach that can be utilized for rapid characterization of organic nitrogen bases. Because of the extreme complexity of diesel oils, no single analytical technique can provide the identification of the position of the nitrogen atom in individual azaarene isomers. Although mass spectrometry is widely used as a structure elucidation tool for petroleum products, it is not very useful in determining the position of the nitrogen atom due to the lack of fragmentation patterns in the mass spectra of molecules having more than two fused aromatic rings[13, 14]. UV spectrometry has been used successfully to identify di- and triaromatic azaarenes[10] and is applied in this work to

differentiate various classes of benzoquinolines. Also discussed is the development of HPLC systems to separate these closely related nitrogen bases and the usefulness of the LC/MS technique as an alternative to conventional GC/MS methods.

MATERIALS AND METHODS

Materials:

The diesel oil sample was provided by Petrobras of Brazil. It was laboratory distilled at Petrobras and had a boiling point range of 200 - 400 °C. The total nitrogen content of this sample was approximately 700 ppm. Neutral aluminum oxide powder was obtained from J.T. Baker, Inc. and was of chromatography grade. Reference standards of benzo[h]quinoline and acridine were purchased from Aldrich Chemical Company and were analytical grade. Chemical structures of benzo[h]-, benzo[f]quinoline, and acridine are shown in Figure 1. All other chemicals were obtained from commercial sources and were at a minimum reagent grade. All solvents were HPLC grade.

Fractionation of basic nitrogen compounds:

Experimental procedures for the fractionation of basic nitrogen compounds in the Brazilian diesel sample are detailed in our previous work[12]. Briefly, approximately 500 mL of the diesel oil sample was dissolved in 500 mL of hexane and partitioned with 3 X 300 mL of 10% sulphuric acid and then with 300 mL of 20% sulphuric acid. The aqueous acid extracts were combined and washed with dichloromethane. After raising the pH to 12 - 13 with sodium hydroxide, the aqueous fraction was extracted with dichloromethane. The combined dichloromethane extracts were washed with water, dried over anhydrous sodium sulphate, and the solvent was removed by rotary evaporation. This sample was labeled as the basic fraction and an aliquot of it was dissolved in methanol for HPLC separations and/or LC/MS analysis.

High Performance Liquid Chromatography (HPLC):

HPLC instruments included a Hewlett Packard 1050 gradient solvent pump, a Hewlett Packard 1050 autosampler, a Kratos 757 UV detector, and a Hewlett Packard 3396A integrator. LC/MS analyses were conducted using a Hewlett Packard 5989A MS Engine equipped with a particle beam interface. Full scan electron impact (EI) mass spectra were obtained using 70 eV electron energy at a source temperature of 250 °C. HPLC-UV analyses were conducted using

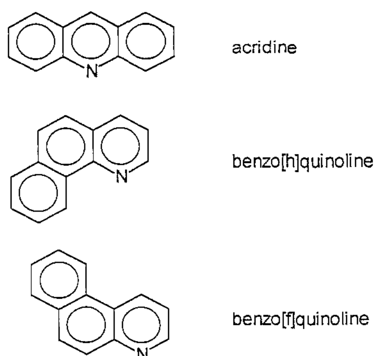

Figure 1. Chemical structures of acridine, benzo[h]quinoline, and benzo[f]quinoline.

online photo diode-array detection (Hewlett Packard 1040A). The UV scan range was 220 to 400 nm.

Two reversed phase chromatographic systems were developed for the separation of the basic fraction. Both systems utilized a MetaChem Nucleosil C18 (5 μm, 150 X 2.0 mm) column with gradient elution at 0.4 mL/minute. System I used neutral mobile phases (A: 40/60 acetonitrile/water; B: acetonitrile). The linear gradient program was: 0 min, 100A; 40 min, 50A/50B; 60 min, 100B. System II used acidified mobile phases (A: 10/90/0.2 acetonitrile/water/acetic acid; B: 80/20/0.1 acetonitrile/water/acetic acid). The linear gradient program was: 0 min, 100A; 60 min, 100B.

Capillary Gas Chromatography (GC):

GC/MS analysis was conducted using a Hewlett Packard model 5890 series II gas chromatograph coupled to a Hewlett Packard model 5971A mass selective detector (MSD). Experimental conditions were:

column:	Restek Rtx-1 (crossbonded 100% dimethyl polysiloxane, 30m x 0.25mm)
carrier gas:	helium
ionization:	electron impact (EI) at 70 eV
acquisition mode:	scan (60 - 300 amu)
temperature program:	initial - 70°C; initial time - 1 minutes; rate - 2°C/minute; final - 250°C; final time - 1 minutes

MSD was turned on 3 minutes after sample injection (solvent delay).

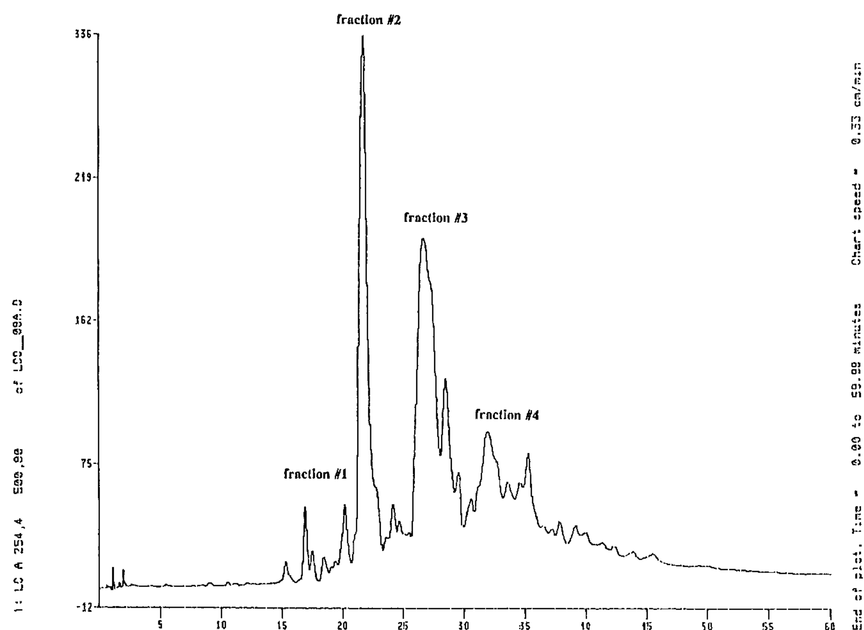

Figure 2. HPLC/UV (254 nm) chromatogram of the basic fraction using chromatographic system I. Also shown is the HPLC isolation scheme.

HPLC isolation of benzoquinoline homologues:

Major components of the basic fraction were isolated by HPLC using a semi-preparative HPLC column (Nucleosil C18, 5 μm, 250 x 10 mm). The isocratic mobile phase consisted of A: 40/60 acetonitrile/water and B: acetonitrile at a ratio of 50%A and 50%B. The flow rate was 5 mL/minute. The isolated fractions were concentrated to small volumes and then partitioned with ethyl acetate. Ethyl acetate extracts were separated, concentrated to dryness, and residues reconstituted in methanol. To check peak purity, the methanol solutions were reinjected onto an analytical C18 column using chromatographic system I.

RESULTS AND DISCUSSION

HPLC separation of benzoquinolines

We reported previously[12] that the basic nitrogen fraction of the Brazilian sample contained mostly alkyl substituted benzoquinoline homologues (C_2- to C_4- benzoquinolines). Similar to the

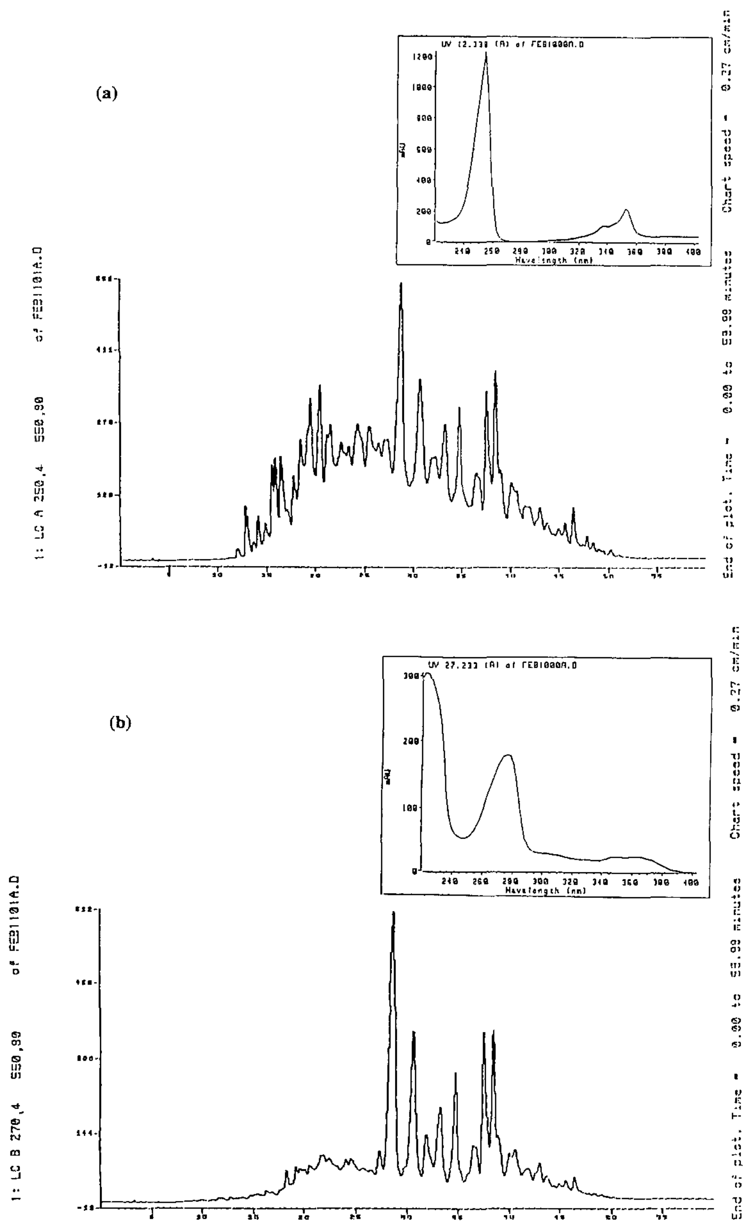

Figure 3. HPLC separation of the basic fraction using acidified mobile phases (chromatographic system II). (a) - 250 nm, (b) - 270 nm.
(insert to (a) - UV spectrum of acridine, insert to (b) - UV spectrum of benzo[h]quinoline)

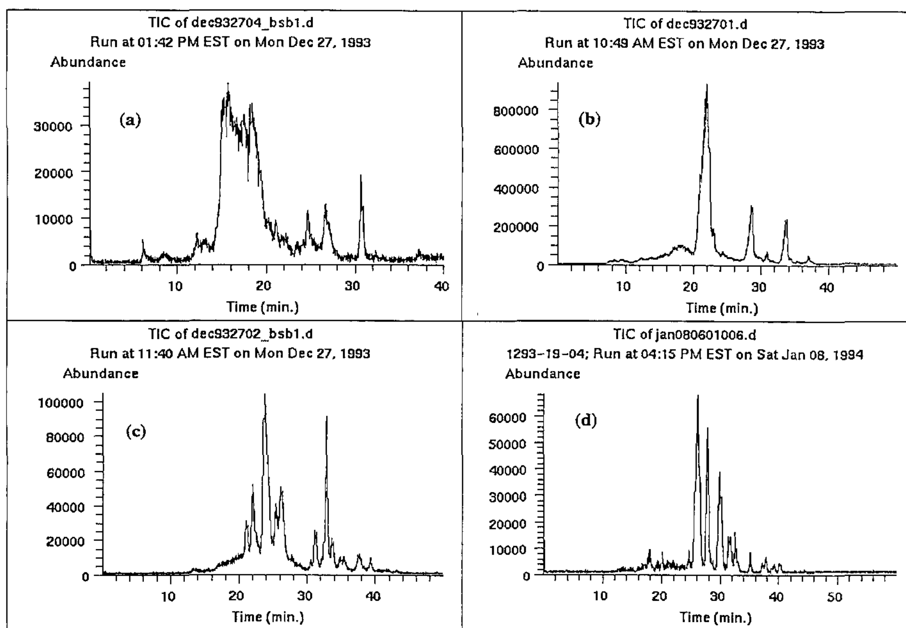

Figure 4. **LC/MS total ion chromatograms of (a) the isolated C_2- acridines (HPLC fraction isolated #1); (b) the isolated C_2- benzo[h]quinolines (HPLC isolated fraction #2); (c) the isolated C_3- benzo[h]quinolines (HPLC isolated fraction #3); and (d) the isolated C_4- benzo[h]quinolines (HPLC isolated fraction #4). Scan range: 70 - 300 amu.**

neutral nitrogen fraction (mostly carbazole homologues)[12], the HPLC separation of the basic fraction using neutral mobile phases was adequate for various benzoquinoline homologues. Using this condition, well resolved benzoquinoline homologues could be readily isolated preparatively for further characterization. However, the resolution of individual isomers using a neutral mobile phase was poor. By using acidified mobile phases, the resolution of individual isomers was greatly improved. Figures 2 and 3 illustrate the effect of acid on the separation of benzoquinolines. Based on the UV signal ratios at 250 and 270 nm (Figure 3), it was evident that this sample contained at least two classes of benzoquinolines - acridines and benzo[h]quinolines. (UV spectra of authentic standards of benzo[h]quinoline and acridine are shown in Figure 3.) The UV identification of benzo[h]quinolines and acridines was also supported by LC/MS. It should

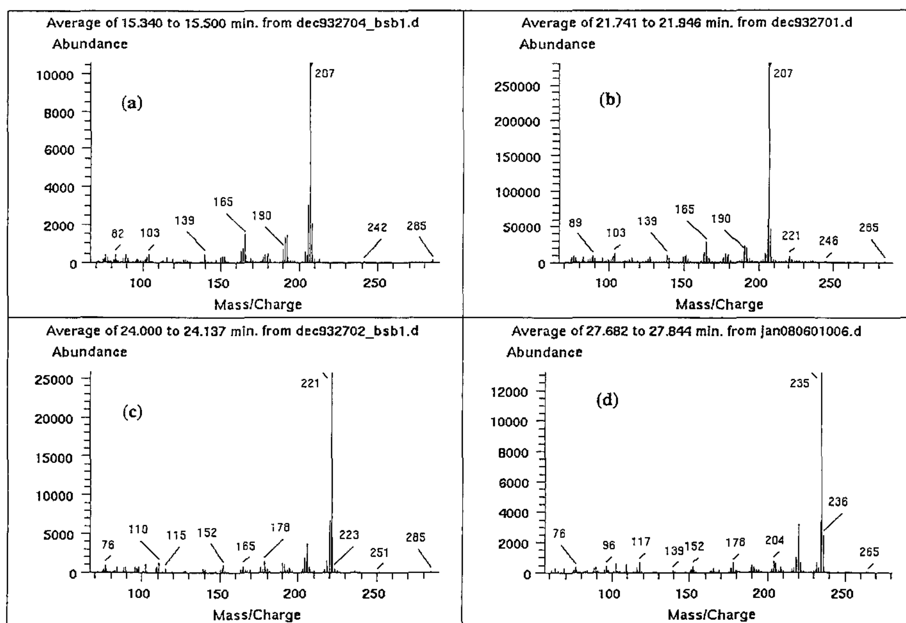

Figure 5. **LC/MS EI mass spectra of (a) C_2- acridine, (b) C_2- benzo[h]quinoline (c) C_3-benzo[h]quinoline, and (d) C_4- benzo[h]quinoline identified in HPLC isolated fractions #1, #2, #3, and #4, respectively.**

be noted that benzo[h]quinolines and acridines showed quite different chromatographic behaviors. Under acidic mobile phase conditions, acridines eluted much earlier than benzo[h]quinolines and showed significantly broader peaks. Since their mass spectra are nearly identical, benzo[h]quinolines and acridines are difficult to differentiate by conventional GC/MS methods. The combination of HPLC with photo diode-array detection and LC/MS enabled the identification of these two different classes of benzoquinolines.

Analysis of individual benzoquinoline homologues

One of our research interests is to study the distribution of individual nitrogen base isomers that are found in petroleum and to explore their geochemical formation pathways[15]. To investigate individual benzoquinoline homologues, major components of the basic fraction were isolated via repetitive HPLC injections on a semi-preparative column. The isolation scheme is shown in

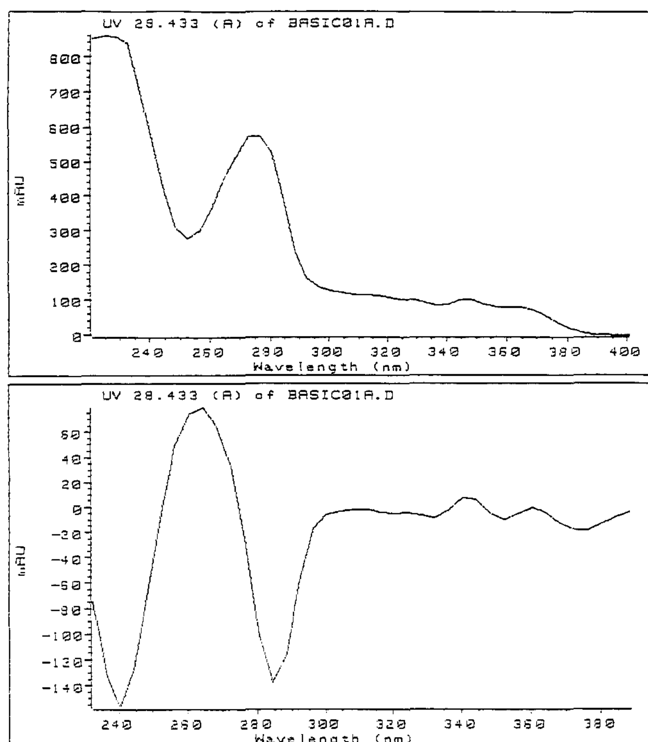

Figure 6. **Top - UV spectrum of a C_2- benzo[h]quinoline isomer identified in the HPLC fraction #2, and Bottom - the first derivative of the absorbance dA/dλ plotted vs. the wavelength λ.**

Figure 2. The isolated fractions were concentrated and analyzed by particle beam LC/MS and HPLC/UV using HPLC system II. Full scan total ion chromatograms of these fractions are presented in Figure 4. Although the mass spectra of C_2- benzoquinoline and C_2- acridine were nearly identical, the fraction #1 (Figure 4a) was identified to be C_2- acridine (m/z 207) based on its UV characteristics. Fraction #2 (Figure 4b) contained three major isomers of C_2-benzoquinoline (m/z 207), while fraction #3 (Figure 4c) exhibited at least eight isomers of C_3-benzoquinoline (m/z 221). Fraction #4 (Figure 4d) was identified as the C_4- benzoquinolines having a similar number of isomers as that of the C_3- homologues. Full scan electron impact mass spectra of representative peaks in each fraction are shown in Figure 5. Based on the low values

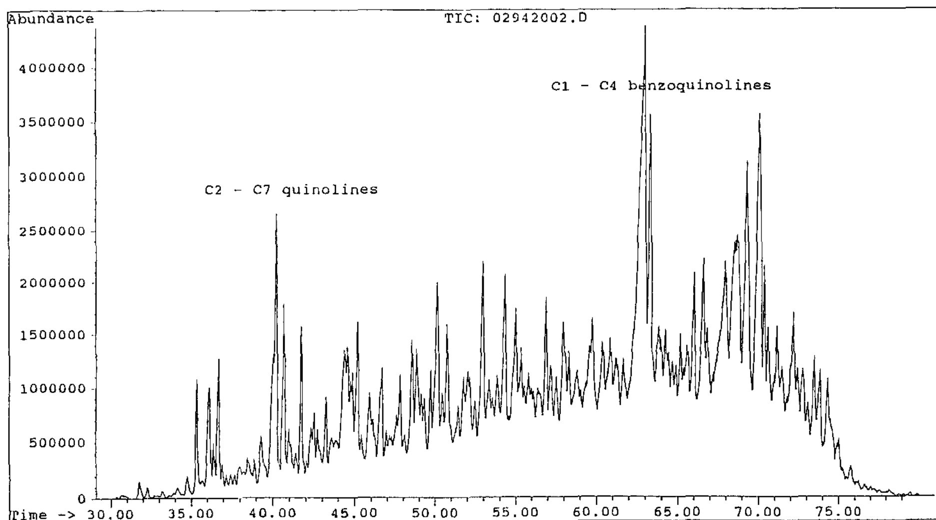

Figure 7. GC/MS total ion chromatogram of the basic fraction showing the separation of quinoline and benzoquinoline homologues. Scan range: 60 - 300 amu.

(e.g. < 0.4) of the two separate ratios of ions $(M\text{-}H)^+/M^+$ and $(M\text{-}CH_3)^+/M^{+1}$, as well as the absence of rearrangement ions, it is likely that the benzoquinolines are polymethylated (rather than ethyl, propyl, or butyl substituted). This conclusion is consistent with previous results[15-19].

The other possible classes of benzoquinolines, besides the benzo[h]quinolines, that might be present are the benzo[f]quinolines. Since their mass spectra as well as UV spectra are almost indistinguishable, the identification of benzo[h]- and benzo[f]quinolines presented significant difficulties. However, by plotting the first derivative of the absorbance ($dA/d\lambda$) vs. the wavelength, the two classes of compounds can be distinquished[20]. The ratio of the absorbance in the vicinity of 240 and 280 nm is close to 1 for benzo[h]quinolines and greater than 3 for benzo[f]quinolines[10]. Thus, this fingerprinting technique can significantly simplify the recognition patterns for isomeric types of azaarene molecules. The usefulness of this technique is illustrated in Figure 6. Based on similar UV data, it was concluded that benzo[h]quinolines were the major components in the HPLC isolated fractions #2 - #4.

GC/MS analysis of the basic fraction

To assist in the characterization scheme and to compare to the particle beam LC/MS technique, the basic fraction was also analyzed by GC/MS using full scan electron impact

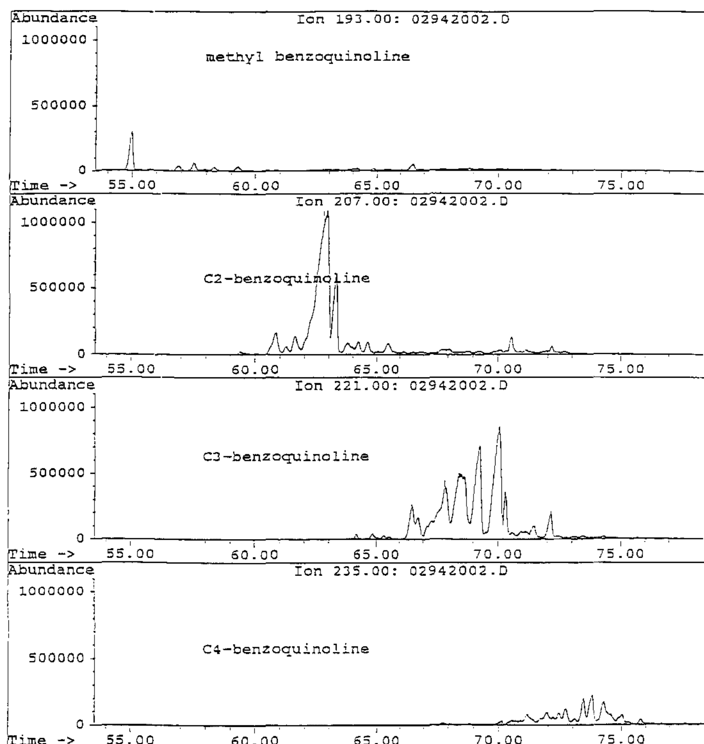

Figure 8. GC/MS extracted ion chromatograms of C_1- to C_4- benzoquinolines (m/z 193, 207, 221, and 235).

ionization. The total ion chromatogram of this sample is presented in Figure 7. Extracted ion chromatograms (m/z 193, 207, 221, and 235) are presented in Figure 8 showing benzoquinoline homologues and the separation of individual isomers. As shown, the separation obtained on a capillary GC column was significantly improved compared to HPLC. The other noticeable difference is the strong signals for the quinoline homologues (C_2- to C_7 -). Extracted ion chromatograms (m/z 157, 171, 185, 199, 213, and 227) are presented in Figure 9. Quinolines were also detected by particle beam LC/MS but at much lower signals compared to benzoquinolines. This was partially attributable to the higher volatility of quinolines which resulted in lower sensitivity by particle beam LC/MS. (Volatile compounds are often pumped away in the interface region prior to reaching the ion source.) Under reversed phase HPLC conditions, quinolines elute earlier than benzoquinolines. HPLC-UV analysis at both 250 and 270

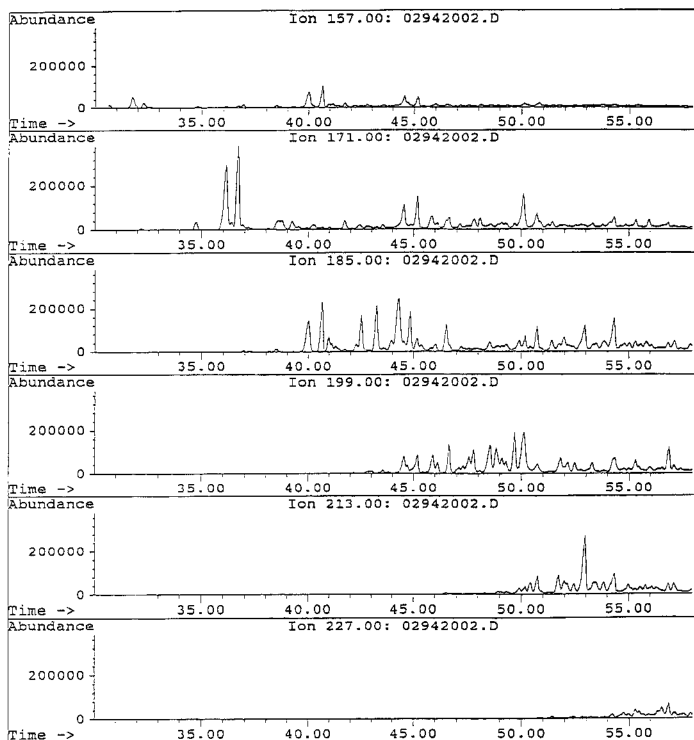

Figure 9. **GC/MS extracted ion chromatograms of C_2- to C_7- quinolines (m/z 157, 171, 185, 199, 213, and 227).**

nm showed that quinolines were minor components in the sample, which supported LC/MS data. On the other hand, GC/MS was much more sensitive to quinolines (because of their high volatilities) and intense signals were acquired. We should point out that under GC/MS conditions nitrogen species probably co-eluted with several polyaromatic hydrocarbons from the sample matrix. As a result, interpretations of some of the mass spectra were difficult. Nevertheless, both techniques (GC/MS and LC/MS) showed their effectiveness in characterizing complex samples such as diesel oils. Because of the direct correlation of LC/MS to HPLC/UV, difficult tasks such as distinguishing different types of alkylazaaerenes can be resolved. Such an advantage clearly places LC/MS among the attractive alternatives to conventional GC/MS methods as well as being a convenient method for direct analysis of some of these complex mixtures[20].

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DETERMINATION OF SULFAMATE AND SULFATE AS DEGRADATION PRODUCTS IN AN ANTIEPILEPTIC DRUG USING ION CHROMATOGRAPHY AND INDIRECT UV DETECTION

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ABSTRACT

Topiramate (bis-O-(1-methylethylidene)-fructopyranose sulfamate) is a potent antiepileptic drug currently in phase III clinical trials. Sulfamate and sulfate have been found to be two stoichiometrically formed degradation products in topiramate. An ion chromatographic method with indirect UV detection has been developed to assay sulfamate and/or sulfate in topiramate drug substance and formulated products. When used in combination with an HPLC assay method, this method is stability-indicating and can be used as a regulatory method.

INTRODUCTION

Traditionally carbohydrates and derivatives are not considered good candidates for pharmacological development. In the last few years, however, new evidence of pharmacological activity for some carbohydrates has excited many scientists in the pharmaceutical industry. Currently, carbohydrates are being investigated by pharmaceutical companies and academic institutes alike in many therapeutic areas including rheumatoid arthritis, ulcer, tumor, tissue

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repair, cardiovascular, and inflammatory diseases [1-3]. Topiramate, a sulfamate derivative of fructose developed by the R.W. Johnson Pharmaceutical Research Institute, is a potent antiepileptic drug currently in Phase III clinical trials [4].

In the process of developing regulatory analytical methodology, the analytical chemists faced several problems that have been proven to be very interesting and challenging. Like most other carbohydrates, topiramate and most related impurities do not have a chromophore active above 190 nm. This limits the choices of detection techniques if an HPLC method needs to be developed. Also, topiramate, the process impurities and degradation products cover a wide range of polarity. This makes it very difficult to select the right column, elution mode and mobile phase which is compatible with the detector. Another problem was to achieve mass balance in the stability studies. Insoluble residues were formed in some stability samples and mass balance was not observed for those samples using conventional HPLC or capillary GC methods by assaying the major and organic degradation products.

This paper describes a simple ion chromatographic method which is stability-indicating when used in combination with an HPLC assay method.

EXPERIMENTAL

Materials

Topiramate drug substance was prepared by the R.W. Johnson Pharmaceutical Research Institute as previously reported [4]. HPLC grade methanol and water were used to prepare the mobile phase. *p*-Hydroxybenzoic acid was obtained from Sigma (St. Louis, Missouri).

Apparatus

An HPLC system consisting of a Waters 600E pump, a WISP 712 automatic injector and an Applied Biosystems 783A programmable UV detector was used. Data acquisition was done using a Hewlett Packard 3357 laboratory automation system via a 18652A A/D converter.

HPLC Conditions

The method utilizes a polymer based anion-exchange column (PRP-X100, 10 µm particle size, 15 cm x 4.6 mm I. D.) purchased from Hamilton (Reno,

Nevada) and indirect UV detection at 310 nm [5]. The mobile phase was a mixture of 5.8 mM *p*-hydroxybenzoic acid and 2.5% methanol and was adjusted to pH 9.4 ± 0.1 using sodium hydroxide. All analyses were performed isocratically at 40 ^{o}C ± 0.1 with a flow rate of 1.5 mL/min. The injection volume was 100 μL.

Sample Preparation

For drug substance, accurately weigh about 40.0 mg sample into a 0.5 oz bottle. Pipette 10.0 mL of mobile phase into the bottle and shake for one hour. Visually inspect each sample solution. If insoluble particles are found in the solution, filter the sample through a 0.45 μm Nylon 66 Whatman filter (Clifton, NJ), discarding the first 5 mL of filtrate.

For tablets (100 mg strength), place 10 tablets into a 250 mL volumetric flask. Add 200 mL of mobile phase into the flask and shake for one hour. Dilute to volume with mobile phase and shake well. Filter each diluted sample through a 0.45 μm Nylon 66 Whatman filter, discarding the first 5 mL of filtrate.

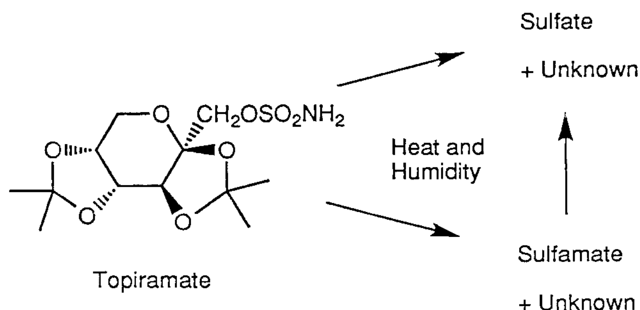
RESULT AND DISCUSSION

The method was validated for monitoring sulfate in topiramate tablets and sulfamate and sulfate in topiramate drug substance. Validation studies, including specificity, solution stability-indicating ability, recovery, linearity, precision, sensitivity, and ruggedness, were performed. The results are summarized in Table 1.

Topiramate (Scheme 1) in the solid state is very stable at ambient temperature. In fact, several batches of topiramate have been stored at room temperature for several years with no noticeable degradation detected. At elevated temperatures , however, degradation was observed for drug substance and formulated products. Interestingly, when degraded samples were assayed by a reversed-phase HPLC method [6], a decrease in topiramate assay values was observed, but no proportional amount of degradation products could be detected. Meanwhile, insoluble black particles were found in the degraded samples. An elemental analysis was performed for one of the degraded samples and the result indicated that black carbon was present which implied that a mass balance would not be achieved by assaying topiramate and the organic moieties of the

Table 1: Selected Method Validation Results

Specificity -	Specificity of this method is determined by resolving sulfamate and/or sulfate from system peaks
Linearity -	The assay response was linear from 0.25 to 18.8 mole percent for sulfate and 0.25 to 6.3 mole percent for sulfamate
Precision -	The method precision (ten replicates), expressed as relative standard deviation (RSD%), was 6.1% for sulfamate at the 0.7 mole percent level and 5.8% for sulfate at the 1.5 mole percent level.
Sensitivity-	The limit of detection was determined to be 0.1 mole percent for both sulfamate and sulfate (signal/noise = 2). The limit of quantitation was determined to be 0.3 mole percent for sulfamate and sulfate with an RSD% $\leq$ 10%.
Solution Stability -	Sample solutions are stable at ambient temperatures for 2 days
Recovery -	The recovery of sulfamate and sulfate from degraded drug substance samples and the recovery of sulfate from degraded tablet samples, both contained insoluble black particles, was determined by exhaustive extraction followed by IC analysis. A range of extraction times from 1 hour to 22 hours was studies and did not affect the recovery.
Ruggedness -	Data generated to study the method ruggedness indicate that the method is rugged. Mobile phase composition and pH, column temperature and length were varied.



Scheme 1. Topiramate Degradation

degradation products. In probe stability studies, it was found that the degraded drug substance and tablets contained considerable amount of acids. For example, a 10 mg/mL suspension of topiramate drug substance sample stressed at 90 °C and uncontrolled humidity for about 19 hours had a pH of 1.9. The initial pH for undegraded samples was about 6. Based on literature references [7] and the above observations, we proposed a degradation pathway for topiramate in Scheme 1. The simplified scheme is used to develop an analytical strategy rather than to describe topiramate degradation.

Degradation studies were carried out to confirm the proposed scheme. Topiramate drug substances were stressed at 70 and 90 °C and uncontrolled relative humidity. Samples pulled at different time points were assayed for sulfamate and sulfate using this ion chromatographic method and for topiramate remainings using a reversed-phase HPLC method [6]. The results are presented in Table 2. Sulfamate and sulfate were detected in the drug substance samples stressed at 90 °C after 11 hours. At 15 hours, the concentration of sulfamate reached a maximum of 5.7% (mole) then started decreasing whereas the concentration of sulfate increased steadily as topiramate assay values decreased. A similar pattern was observed for samples stressed at 70 °C except that the maximum concentration of sulfamate was only 1.3% (mole) observed at 120 and 144 hours.

The mass balance (recovery) data are also presented in Table 2. For the 90 °C samples, the total recovery decreased with time and was 71.0% when about 44.1 % topiramate was degraded. The lack of total recovery with these samples indicated a possibility of several parallel degradation reactions for topiramate at this temperature. The degradation reactions may generate sulfamate, sulfate, black carbon and some unknown organic degradation products to which the sulfamate functionality was still attached. Therefore, Scheme 1 is not a complete description for topiramate degradation at 90 °C. For the 70 °C samples, the improved recovery (96.6% - 98.3%) was time-independent and acceptable for practical purposes. The best recovery results were observed for three batches of degraded topiramate tablets which had been stored in three different containers at 40 °C for 9 months. Different amounts of desiccant were present in two of those containers. These samples were analyzed using this method for sulfamate and sulfate. The method had to be modified to assay sulfamate because of interference from the excipients [8]. It is interesting that

Table 2: Weight Percent Assay Values for Topiramate and Degradation Products Observed in Stressed Drug Substance and Tablets

	Stress Cond. °C/hour	Sulfamate (mole%)	Sulfate (mole%)	Topiramate (%)	Recovery (%)
Drug Substance					
	90/7	0.0	0.0	100.4	100.4
	90/11	0.4	0.2	98.7	99.3
	90/13	2.0	0.5	91.6	94.1
	90/15	5.7	3.5	72.1	81.3
	90/17	4.0	11.1	55.9	71.0
	70/120	1.3	2.0	93.9	97.2
	70/144	1.3	6.4	90.0	97.7
	70/168	0.6	9.7	86.3	96.6
	70/192	0.5	10.4	87.4	98.3
	70/240	0.9	13.8	83.1	97.8
Tablets					
	40/9 month[a]	N/A	13.4	86.3	99.7
	40/9 month[b]	N/A	7.3	93.0	100.3
	40/9 month[c]	N/A	0.7	100.7	101.4

a - No desiccant in container
b - 1 g desiccant in container
c - 10 g desiccant in container

only sulfate was detected in these samples. and that the stability of topiramate is related to the amount of desiccant present in the containers.

In summary, for topiramate drug substance and tablets exposed to elevated temperatures ($\leq$70 °C), sulfamate and/or sulfate are the stoichiometrically formed degradation products. Therefore, the assay of sulfamate and/or sulfate is equivalent to assaying the total organic moieties of degradation products that do not contain sulfur. This strategy takes advantage

of the fact that sulfamate and sulfate are stable and nonvolatile chemical entities, while the disadvantage is that two assay methods are needed (one for the active drug and one for the degradation products) for the release testing and stability monitoring of the drug substance and tablets. Currently at the R. W. Johnson Pharmaceutical Research Institute, this strategy has been incorporated into the official stability testing program for topiramate. Many drug substance and tablet samples stressed at 40 or 50 $^{\circ}C$ have been assayed. Good mass balance has been observed for all samples assayed.

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LIQUID CHROMATOGRAPHIC ASSAY FOR THE SEPARATION OF SINGLE- AND DOUBLE-STRANDED DNA BY USING UV AND UV DIODE-ARRAY DETECTORS AND HYDROXYLAPATITE COLUMN

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method, using UV and UV diode-array (DA) detection, is reported for the separation of single-stranded (s.s.) and double-stranded (d.s.) DNA molecules. Commercially available calf thymus DNA was used as the standard, to develop and optimize necessary analytical procedures and chromatographic parameters. Bio-Gel® hydroxylapatite was used as the column packing and the sorbed polynucleotides on the column matrix were separated by using an ionic strength gradient system consisting of phosphate buffer at pH 6.8. The stationary phase was stable and proved sufficiently reliable in the separation and resolution of s.s. and d.s. DNA molecules in the standard. Pointedly, the DA detector was more sensitive to the analytes than the UV detector. The response of both detectors was higher for the s.s. DNA compared to the d.s. DNA. Minimum quantification limits (MQL) for the s.s. DNA molecules by the DA and UV detectors were, respectively, 0.10 and 0.50 µg in 10 µL injections. The corresponding value for the d.s. DNA, using both detectors, was 1.0 µg. The plot log (µg of DNA) *vs* absorbance (mAU) was linear for the d.s. DNA. The MQL, using both detectors, was 0.10 µg in 10 µL injection volume. Extension of the method to separate the viral DNA molecules showed some promise. However, problems associated with sample purity and homogeneity, peak characterization, quantification of the analytes etc. were encountered and these drawbacks are discussed.

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INTRODUCTION

The use of high-performance liquid chromatography (HPLC) is gaining importance in the separation and analysis of nucleic acids over the conventional electrophoresis method because of its reproducibility and quantitative accuracy (1,2). The introduction of several novel column packing materials enhanced the isolation and analysis of nucleic acids and their fragments with excellent resolution (3-9). In normal phase chromatography, organic resins used previously have been replaced by microparticle silica gels, improving resolution, sensitivity and speed (2). On the reverse phase columns, the introduction of alkylated supports, such as C_8 or C_{18} bonded nonporous silica gel and silica gel-based weak anion and cation exchangers, is finding extensive use in the separation process of different types of biopolymers (2).

The use of microcrystalline hydroxylapatite $[Ca_{10}(PO_4)_6(OH)_2]$ as stationary phase in HPLC has been documented earlier and used for the rapid and quantitative separation and purification of nucleic acids (10-12). The analyte molecules bind to hydroxylapatite by electrostatic interactions between the Ca^{2+} ions in the stationary phase and PO_4^{3-} ions in the polynucleotides (13). Elution of the analyte is achieved by the competition of PO_4^{3-} ions in the eluate buffer, for hydroxylapatite binding sites (14).

Recently, we investigated the separation patterns of s.s. and d.s. nucleic acid components of a denatured nuclear polyhedrosis virus (NPV), isolated from the insect pest, gypsy moth (*Lymantria dispar* L.) (GM), by using a high-resolution hydroxylapatite column linked separately to two liquid chromatographs, one containing an ordinary UV detector and the other a UV diode-array (DA) detector. Prior to the injection of GM-NPV solutions, we used commercially available denatured calf thymus DNA, containing both single-stranded

(s.s.) and double-stranded (d.s.) DNA, as standard (15) to establish the necessary experimental conditions and instrumental parameters required for the successful completion of the experiment. Results of this study are reported in this paper.

MATERIALS AND METHODS

Chemicals and Reagents

DNA Standard

Native, heat-denatured calf thymus DNA (50.0 mg/mL), containing both s.s. and d.s. DNA, was obtained from Boehringer-Mannheim (201 Boulevard Armand Frappier, Laval, Quebec, Canada, H7V 4A2). A stock solution containing 5.0 mg/mL was prepared in 0.50 M sodium phosphate buffer (pH 6.80 ± 0.05) containing 0.02 % sodium azide and 0.01 mM calcium chloride. The standard solutions, ranging in concentration from 10 μg/mL to 500 μg/mL, were prepared by the serial dilution of the stock solution using deionized Milli-Q® purified water.

GM-NPV DNA

Two samples (labeled as X and Y) of denatured and purified DNA isolated from the NPV of GM, containing 0.043 mg/mL (X) and 0.066 mg/mL (Y) in 0.4 M sodium phosphate buffer, were used in this study after filtering through Millipore® 0.20 μm filters.

Mobile Phase

The mobile phase solvents, A and B, used in HPLC were phosphate buffers (pH 6.80 ± 0.05) containing HPO_4^{2-} and $H_2PO_4^-$. The 0.5 M buffer (B) was prepared according to the procedure of Efiok (16) by using equimolar quantities of ACS grade Na_2HPO_4 and NaH_2PO_4,

and deionized Milli-Q® purified water. The 10 mM buffer (A) was prepared by diluting aliquots of buffer B fifty times with Milli-Q®purified water. The pH of the buffers were checked with a pH meter and if slight adjustments were required, they were done by adding, dropwise, concentrated HCl or NaOH. Each buffer also contained 0.02% NaN_3 and 0.01 mM $CaCl_2$. They were filtered (Millipore® 0.20 μm filter) and degassed prior to use and refrigerated immediately afterwards. No detectable deterioration in DNA resolution of the standards occurred with monthly aged buffers; nevertheless, fresh preparations were made every two weeks as a precaution.

HPLC Instrumentation

Two liquid chromatographs, Hewlett-Packard (HP) Model 1080 fitted with a UV variable wavelength detector and HP Model 1090 fitted with a DA detector, were used in the study. Both instruments had Rheodyne® injectors equipped with 10 to 100 μL loops and computing facilities to extract the necessary analytical data from the chromatograms generated. Full descriptions of these two instruments are published elsewhere (17, 18). The parameters used for the two instruments were similar. A flow rate of 1.0 mL/min was used and the adsorbed DNA was eluted and separated by the stepwise use of a binary gradient system consisting of two phosphate buffers, one with low (buffer B, 10 mM) and the other with high (buffer A, 0.5 M) ionic strength (14). The UV and DA detectors were set at 260 nm (4 nm bandwidth) for the sample wavelength and 400 nm (50 nm bandwidth) for the reference wavelength. The column used was a Bio-Gel® HPHT column (100 × 7.8 mm, 4.8 mL bed volume) packed with Bio-Rad's hydroxylapatite, preceded by a guard column (50 × 4.0 mm) (both columns from BioRad Labs Canada Ltd., Mississauga, Ontario, L4W 2A6) containing inert hydrophilic polymer particles of 10 μm size. The column was thoroughly equilibrated

in the starting buffer prior to sample injection. The oven temperature and column pressure were maintained at 50°C and approx. 1.4×10^4 kPa, respectively, and the total run time for each injection was 20 min.

The ionic strength of the buffer was increased, stepwise, by starting initially at 40% of buffer B for 3 min, increasing it to 45% for the next 4 min and then switching completely to 100% B for the remaining 13 min. Most of the instrumental parameters and operating conditions listed in this study were painstakingly arrived at by trial-and-error, and eventually optimized to attain precision and accuracy in measurements.

HPLC Analysis

During calibration of the instruments using calf thymus DNA as standard, 10 μL volumes, containing 0.1 to 5.0 μg of the analyte, were injected (in triplicate) in each instrument and the corresponding detector response was recorded. Calibration curves were prepared for the s.s. and d.s. DNA by plotting the average peak area (y-axis) against the mass of DNA injected (x-axis). The standard deviation (SD) of the peak area for each concentration at 1.0 μg in 10 μL and above was found to be roughly proportional to its mean, however this was not the case for concentrations below the 1.0 μg/10 μL level, wherein the SD was high. The average retention times (RTs) for the s.s. and d.s. calf thymus DNA were found to be 8.1 (range 8.0 to 8.2) and 11.9 (range 11.7 to 12.1) min, respectively.

Aliquots of the two test solutions, X and Y, containing the GM-NPV DNA were then injected and the peak areas were computed from the chromatograms obtained. Unfortunately, none of the RTs of the peaks obtained corresponded to those of the standards. The peaks with RTs 10.1 and 15.3 min in sample X and 10.8 and 15.9 min in

sample Y, of the GM-NPV, were assumed to correspond to the s.s and d.s. DNAs, respectively. Their relative concentrations in the two samples were computed using the calibration curves generated for the calf thymus standard.

RESULTS AND DISCUSSION

Response of UV and DA Detectors to DNA Standard

Under the HPLC parameters used and from the calibration curves (Figure 1), it is apparent that both the UV and DA detectors responded satisfactorily to the s.s. and d.s. DNA molecules. It is evident that the response of the DA detector to the s.s. DNA was linear over the concentration range of 0.1 to 5.0 μg in 10 μL injection volume. However, the response of the UV detector to the s.s. DNA was generally low and was linear only from 0.5 to 5.0 μg. Below 0.5 μg, the detector response was rather erratic. Within these ranges, the reproducibility of peak area measurements in both detectors was 93.5% as determined by 10 repeat injections. The linear regression equations for the two calibration curves for the s.s. DNA, from the DA and UV detectors, and the correlation coefficients (R^2) for them are:

DA detector: s.s. DNA $y = 1053x - 14.70$, $R^2 = 0.999$

UV detector: s.s. DNA $y = 692.7x - 141.6$, $R^2 = 0.989$

From the standard curves in Figure 1, it is apparent that the DA detector is more sensitive (higher slope) to the s.s. DNA than the UV detector. The minimum quantification limits (MQL) for the s.s. DNA standard in the DA and UV detectors, as assessed from the respective standard curves, were 0.10 and 0.50 μg, respectively.

The standard curves obtained (Figure 1) for the d.s. DNA using the DA and UV

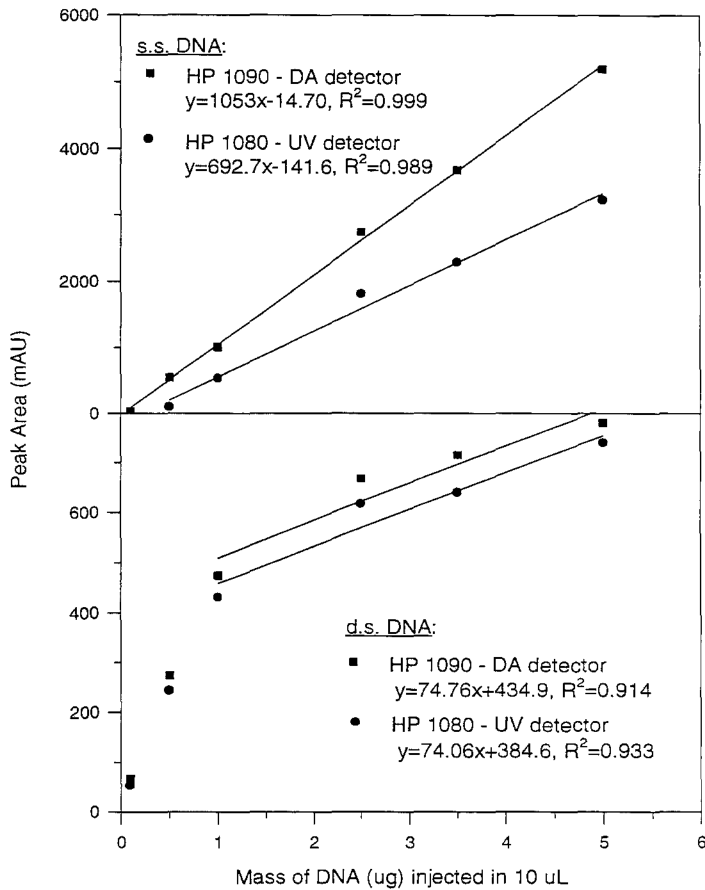

FIGURE 1. Calibration of HP 1080 and HP 1090 using calf thymus DNA - single and double strands.

detectors were not overly influenced by the analyte concentration. Concomitantly, the response of both detectors was very low, indicating their low sensitivity to the d.s. DNA. Linearity of the detectors, by plotting μg of DNA *vs* absorbance (mAU), was observed only over the concentration range from 1.0 to 5.0 μg in 10 μL injection volume. Moreover, the standard curves did not pass through the origin and their slopes were low. The reproducibility of peak area measurements was about 91% as determined by 15 repeat injections. The linear regression equations for the two calibration curves for the d.s. DNA, from the DA and UV detectors, and the corresponding correlation coefficients are:

DA detector: d.s. DNA $y = 74.76\,x + 434.9$, $R^2 = 0.914$

UV detector: d.s. DNA $y = 74.06\,x + 384.6$, $R^2 = 0.933$

The MQL established for the d.s. DNA standard, from the calibration curves of both detectors, was 1.0 μg.

The plot of log (μg of DNA) *vs* absorbance (mAU) was linear for the d.s. DNA, for both detectors over the concentration range of 0.10 to 5.0 μg in 10 μL injection volume. The linear regression equations and corresponding correlation coefficients are:

DA detector: d.s. DNA $y = 438.1\,x + 471.3$, $R^2 = 0.985$

UV detector: d.s. DNA $y = 412.4\,x + 430.6$, $R^2 = 0.982$

The MQL for the d.s. DNA standard, using linear regression equations from plotting log (μg of DNA) *vs* absorbance (mAU), was 0.10 μg in 10 μL injection volume for both detectors.

HPLC Chromatogram of the DNA Standard

A typical chromatogram of the calf thymus DNA standard, obtained by injecting (in triplicate) 2.5 μg in 10 μL onto the hydroxylapatite column connected to the HP 1080 and

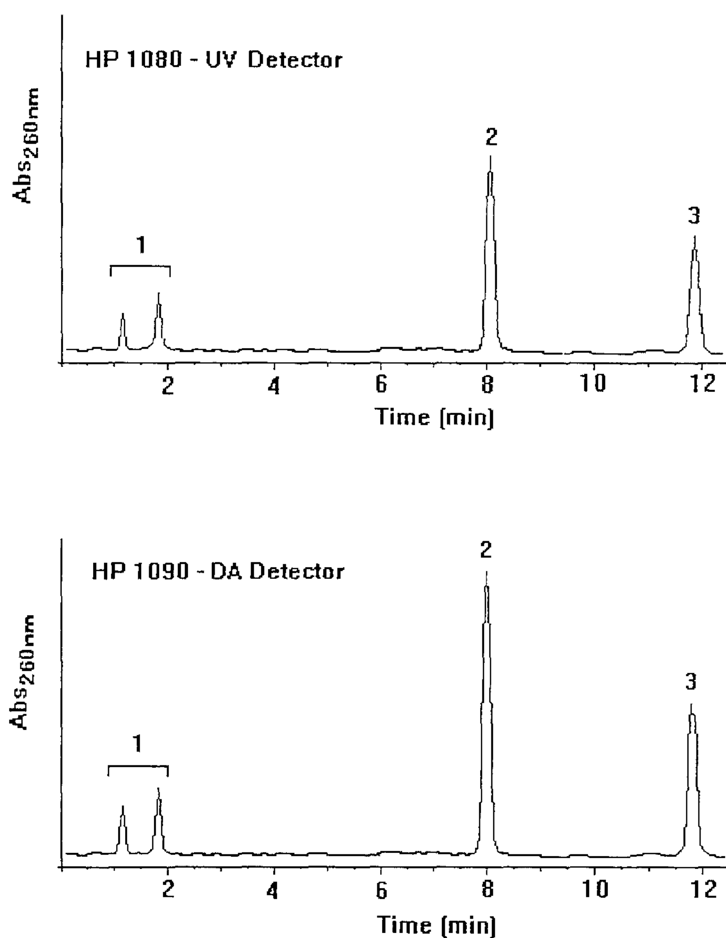

FIGURE 2. Chromatographic trace of calf thymus DNA standard, 2.5 μg and 10 μL injection. Peak 1: low molecular weight materials; peak 2: s.s. DNA (RT, 8.1 min); peak 3: d.s. DNA (RT, 11.9 min). Chromatographic conditions are given in the text.

HP 1090 liquid chromatographs, is shown in Figure 2. As afore-mentioned, the DA detector in HP 1090 HPLC was more sensitive to the s.s. DNA (peak area 2740 $\pm$ 45 mAU, n=3) than the UV detector in HP 1080 instrument (peak area 1812 $\pm$ 50 mAU, n=3). In contrast, the response of both detectors to the d.s. DNA was nearly the same, yielding a similar degree of resolution and sensitivity. This is further corroborated by comparing the peak areas obtained in both instruments. The peak area in HP 1090 for the standard (d.s. DNA) was 667 $\pm$ 40 mAU, whereas in HP 1080, it was 622 $\pm$ 32 mAU.

The chromatographic peaks corresponding to the s.s. (RT = 8.1 min) and d.s. (RT = 11.9 min) DNA molecules in the standard are well resolved and symmetrical. The low RT of the s.s. DNA is presumably the consequence of weak interactions between the Ca^{2+} ions in the stationary phase (hydroxylapatite) and shielded PO_4^{3-} ions in the flexible, disordered and sterically hindered s.s. DNA molecules (19). On the contrary, the higher RT of the d.s. DNA (11.9 min) is most likely due to the unfolding and derotating of the rigid and ordered double helix, which resulted in exposing more PO_4^{3-} ions for strong interaction and binding with Ca^{2+} ions on the sorbent surface. Because of the resultant strong electrostatic interaction, the d.s. DNA molecules are eluted later, giving a higher RT (19). The two small peaks appearing close to the solvent front could be due to low molecular weight nucleotides.

Chromatograms of the Viral DNA

Initial injections of samples X and Y, at 40 to 70 µL range, in both HPLCs, did not produce consistent detector responses and the peak area measurements varied considerably. Increasing the injection volume to 100 µL gave reduced fluctuations and reasonably consistent detector responses. Typical chromatograms obtained in HP 1090 are

shown in Figure 3. The chromatograms contained three large peaks and a number of small peaks of different sizes and shapes, indicating the heterogeneity of DNA moieties and the presence of UV-absorbing impurities in the two samples. The distinct separation of the large peaks indicated that the instrumental and operational parameters used to separate the DNA and other components in the samples were reliable and could be pursued in the future, with necessary modifications, for the purposes of isolation, separation and purification of DNA-type biopolymers.

Examination of Figures 2 and 3 show that none of the peak RTs in Figure 3 matched with the peak RTs of DNA standard in Figure 2. Furthermore, the RTs of the peaks and their shapes (narrow *vs.* broad) in sample X and Y (Figure 3) are different from one another and none of them matched, indicating that the composition of the DNA moieties in the two samples are probably different. The two large peaks (RTs, 2.5 and 2.9 min in Figure 3) near the solvent front, could be due to low molecular weight DNA fragments (20). The next two smaller peaks are likely caused by the partly denatured and hybrid nucleic acid molecules, which displayed low affinities for hydroxylapatite (19). Comparing the chromatograms of the DNA standard (Figure 2), we assume that the peaks with RT of 10.1 min (sample X) and 10.8 min (sample Y) (Figure 3), could correspond to the s.s. viral DNA molecules. We are making this assumption, of course, with very little experimental evidence, aside from the fact that elution behavior is: (1) controlled by the interaction between the Ca^{2+} and PO_4^{3-} ions of the adsorbent and adsorbate, respectively; and (2) separation, as aforementioned, is size- and strand-dependent. Similarly, the peaks with RTs of 15.3 min (sample X) and 15.9 min (sample Y) (Figure 3) are assumed to belong to the d.s. viral DNA molecules, although as pointed out above, we have very little experimental basis for these

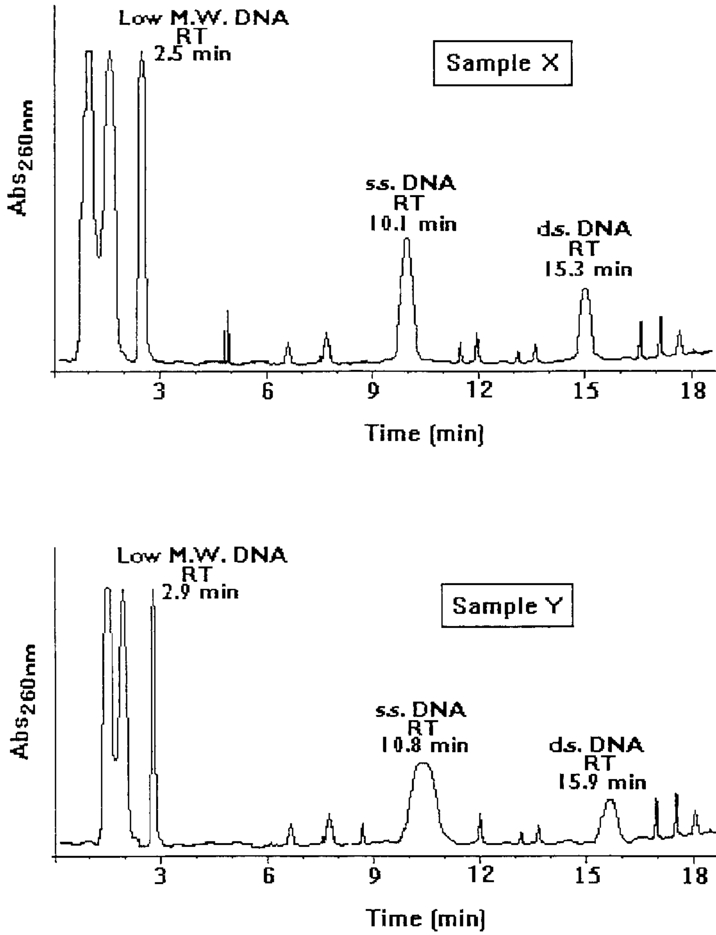

FIGURE 3. Chromatographic trace of viral DNA after injecting 100 μL sample, using the HP 1090 equipped with the DA detector.

assumptions, albeit the appearance of two well defined peaks (narrow in sample X and broad in sample Y) around the elution times of the standards, shown in Figure 2.

Using the calibration curves for the DNA standard in Figure 2 and assuming that the two pairs of peaks with RTs of 10.1 and 15.3 min (sample X), and 10.8 and 15.9 min (sample Y), shown in Figure 3, are due to the s.s. and d.s. viral DNA molecules, respectively, we calculated their concentrations in the two samples. The values obtained in HP 1090 were: s.s. viral DNA, 0.61 μg/100 μL and d.s. viral DNA, 0.70 μg/100 μL; or total viral DNA of 13.1 μg/mL in sample X. The values obtained for sample Y were: s.s. viral DNA, 1.29 μg/100 μL, and d.s. viral DNA 1.39 μg/100 μL; or total viral DNA of 26.8 μg/mL. These values are low and corresponded to only 30.5% and 40.6% of the total viral DNA concentrations expected to be present in the samples X and Y, respectively. Similar calculations, using the peak areas obtained for the analytes in HP 1080 HPLC (chromatograms not shown in figure), gave concentrations which were about 20% less than the HP 1090 values.

CONCLUSIONS

This paper describes a practical and sufficiently sensitive HPLC method to isolate and separate the s.s. and d.s. DNA molecules present in calf thymus DNA standard by using Bio-Gel hydroxylapatite column and phosphate buffer at pH 6.8 as the mobile phase. The column was stable and proved reliable in the separation of DNA molecules. Extension of the method to separate the s.s. and d.s. DNA molecules in two viral DNA preparations yielded chromatograms containing more than seven peaks with different RTs, peak shapes and peak areas for both samples. This indicated not only the heterogeneity in the DNA

composition, but also of the variability in their concentration. Quality maintenance and optimization of methods used in the isolation and preparative purification of the s.s. and d.s. DNA molecules from viral preparations could yield consistency in product excellence, consequently resulting in good chromatographic resolution and analyte separation. Nevertheless, the study succeeded in demonstrating the suitability of the method to isolate and separate nucleic acid moieties found in biological samples. Lack of authentic standards for the viral DNA molecules precluded the absolute quantification of the analytes in the two viral preparations.

ACKNOWLEDGEMENT

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CHROMATOGRAPHIC STUDIES ON THE RACEMIZATION OF THIOPEPTIDES

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ABSTRACT

It was found by chromatographic, CD and NMR methods, that the thionation of piperazine-2,5-diones [*cyclo*(Aaa[1]-Aaa[2]) → *cyclo* (Aaa*t*[1]-Aaa*t*[2] (Aaa = -NH-CHR-CO-; Aaa*t* = -NH-CHR-CS-)] or piperazine-2,5-onthiones [*cyclo*(Aaa*t*[1]-Aaa[2]) → *cyclo* (Aaa*t*[1]-Aaa*t*[2])] and, occasionally, even the spontaneous cyclization of endothiodipeptide esters [H-Aaa*t*[1]-Aaa[2]-OR] result in enantiomeric (Aaa[1] or Aaa[2] = Gly) or diastereomeric mixtures of piperazine monothiones or dithiones. The diastereoisomers were separated by semipreparative HPLC and their quantitative product distribution was determined by an optimized HPLC method on Hypersil-silica column with CH_2Cl_2-EtOAc eluent mixtures. Isocratic RP-HPLC on ODS-Hypersil column and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were used to monitor the racemization of Ala and Pro residues and to de-

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termine the ratio of enantiomers. Thionation of urethane protected dipeptide esters or dethionation of the corresponding endothiodipeptide derivatives were not found to result in significant racemization. However, during the thionation of cyclic dipeptides or thiopeptides or isolation of piperazine-2,5- mono- or dithiones a partial or complete racemization could always be detected. Moreover, the acidic hydrolysis of thiopeptides was also accompanied by racemization and resulted in partially racemized amino (oxo)acids.

INTRODUCTION

There is a growing interest in peptides containing one or more thioamide groups (1-10). Lawesson's reagent (LR) can be used for the conversion of amides into thioamides under mild conditions [heating in benzene or toluene at 30-80°C for 0.5-2 hrs] (9, 11-13). In an effort to study the chiroptical properties, and H-bond forming ability of thiopeptides, a great number of protected endothiopeptides and thionated acyl amino acid and dipeptide methylamides have been prepared in our laboratory (13-19). They were characterized chromatographically by TLC, LC and HPLC; by ^{1}H and ^{13}C NMR, UV, CD and IR spectroscopy, and also by MS (13-19). It has become generally accepted that LR converts the *trans* (Z) rotameric form of the amide group of urethane protected dipeptide esters to the corresponding thioamides selectively and without significant racemization (9, 13, 20).

Based on preliminary CD spectroscopic studies, the thionation of piperazine 2,5-diones (I), featuring *cis* (E) amide groups re-

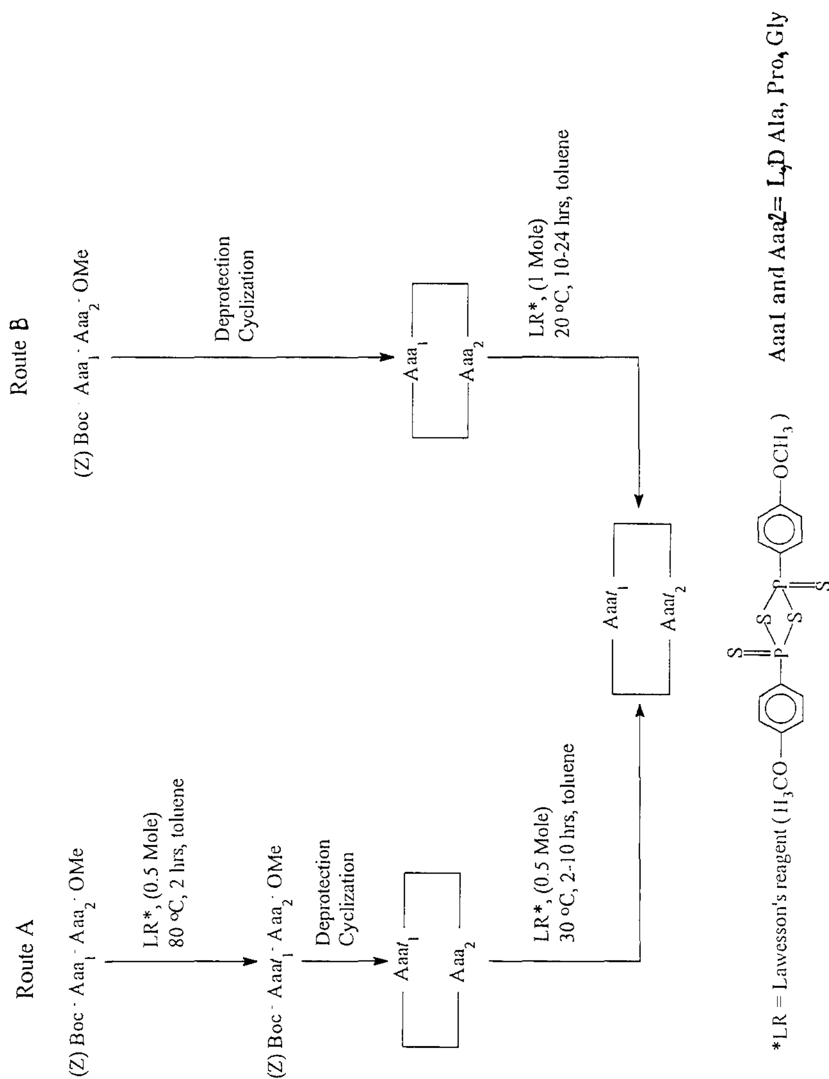
sults, however, in partially or fully racemized piperazine-2,5-dithiones (14, 16).

A violet-coloured crystal of *cyclo*(thioprolyl-thioprolyl), [*cyclo*(Pro*t*-Pro*t*), (III)]; prepared from optically pure L-proline diketopiperazine [*cyclo*(L-Pro-L-Pro)] has been reported to show crystallographic disorder (24) which was explained by the presence of co-crystallized enantiomorphous pairs in the sample (24). Comparative circular dichroism (CD), as well as ^{1}H and ^{13}C NMR studies have indicated that the sample, in addition to the L,L form (*ca.* 80%) contained *ca.* 20% enantiomeric (D,D) but no diastereomeric (L,D) form (14, 24).

Prompted by these findings, a number of single- and double-thionated diketopiperazines have been prepared (14, 16) to clarify the mechanism and conditions of their racemization. Herein we report results of comparative chromatographic studies on the racemization of linear and cyclic thiodipeptides and their non-thionated linear precursors.

MATERIALS

Starting from Z- or Boc-protected linear endothiopeptide esters, (13, 14) a series (Table IV) of piperazine-2,5-onthiones and 2,5-dithiones has been synthesized according to Scheme I. (14) 400 MHz ^{1}H NMR and IR spectroscopy was used to characterize the cyclic thioamides. Details of the syntheses have been reported earlier (13, 14, 16).



Scheme 1.

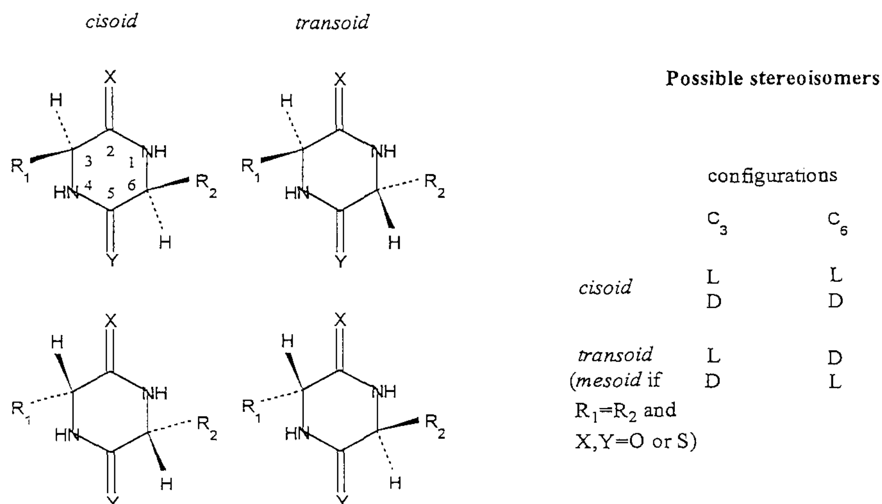
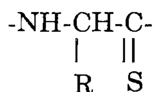


Figure 1. Possible stereoisomers of piperazine 2,5-onthiones (X = O, Y = S), 2,5-thion ones (X = S, Y = O) and 2,5-dithiones (X, Y = S).

The abbreviations used follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (25). Aaa*t* (e.g. Alat, Glyt, etc.) means a thioamino acid residue:



METHODS

High Performance Liquid Chromatography

Separations were performed on a laboratory-assembled instrument consisting of a reciprocating piston pump (Model 1515,

Orlita, FRG), a variable wavelength UV monitor fitted to an 8 μl flow-cell (Model 212, Cecil, UK) and a sample injector (Rheodyne, USA), or on a Knauer HPLC-system consisting of two pumps Model 64 with analytical or preparative pumpheads, a gradient programmer Model 50 B, an injection valve with 20 μl sample loop and a spectral photometer with analytical and preparative flow cells (Knauer-GmbH, FRG).

Column effluents were monitored at 250, 254, 270, 279 or 281 nm (for thiopeptides) and at 340 nm (for Marfey's amino acid derivatives).

The packing materials were Hypersil-silica, Hypersil ODS-6 (Shandon Southern Products, UK), LiChroprep-silica (Merck, FRG) and Partisil M-9 silica (Whatman Ltd, UK). Peaks were recorded on a Model OH-314/1 chart recorder (Radelkis, Hungary) and the areas under them were calculated using programmed Simpson's rule. The chromatographs were operated isocratically with flow rates between 0.8 and 1.2 cm^3/min (analytical mode) and between 2.0 and 4 cm^3/min (preparative mode) at ambient temperature.

Hydrolysis

The linear protected and cyclic thiopeptides were subjected to acidic hydrolysis. The samples were treated at 105°C with 6 M hydrochloric acid for 48 hrs in sealed tubes. The acid was removed in vacuo, samples were neutralized and reacted with Marfey's reagent.

Derivatization

Derivatization was carried out according to Marfey (26) with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce, USA). The hydrolysate prepared from 2-5 μmol starting thiopeptide was dissolved in 100 μl of 0.5 M $NaHCO_3$ solution and 200 μl of 1% solution of Marfey's reagent in acetone was added. The solution was incubated at 40°C for 90 min and cooled; then 25 μl of 2 M HCl was added at room temperature. After 20 fold dilution with methanol or eluant, 10-20 μl aliquots were used for HPLC injection on Hypersil ODS-6 column (27-28).

NMR studies

^1H NMR measurements were performed on a VARIAN 400 spectrometer at ambient temperature, c = 4-8 mg/mL. Solvents: DMSO-d_6 and $CDCl_3$. The assignment of the peaks is based on ^1H-^1H COSY experiments. Data are summarized in Table VIII.

CD measurements

CD spectra were recorded on Jobin-Yvon Dichrographs Mark III and V. D. Mark III is operated by an IBM AT computer. Spectograde solvents (Uvasol, E. Merck, Darmstadt) were used. Measurements were taken in 0.02-1.00 cm cells.

RESULTS AND DISCUSSION

Racemization is one of the major side reactions that may occur during peptide synthesis or in solution in the presence of bases (29). Piperazine-2,5-onthiones or -thionones and 2,5-dithiones, built up from two different chiral amino acid residues ($R_1 \neq R_2 \neq H$) have four stereoisomeric forms (Fig. 1). The number of stereoisomers is decreased by structural factors (incorporation of one glycine or two identical residues). However, steric factors may also lead to the decrease of stereoisomers. For example, the dithione *cyclo*(Prot-Prot), similarly to the parent dioxopiperazine *cyclo*(Pro-Pro), cannot exist in L, D *meso* form (24). Contrary to this, *cyclo*(Ala*t*-Ala*t*) was found to be present as a roughly 1:1 mixture of the enantiomeric (L, L + D, D) and mesoid (L, D) forms (Table II.). Preliminary CD spectroscopic and theoretical studies (30) have indicated that it is the enhanced tendency for thione → thiol tautomerisation of the piperazine-2,5-onthiones or 2,5-dithiones which explains the racemization of cyclic thioamides (30).

The strategy of the synthesis of piperazine-2,5-onthiones and 2,5-dithiones is shown in Scheme 1. The crude products were first pre-purified by chromatography on Kieselgel 60 columns using dichloromethane-ethylacetate mixtures (95:5, 98:2 v/v) as eluant. The diastereomeric mixtures were separated (see Table I) by semipreparative and preparative HPLC and their product distribution was determined (see Table II) by an optimized HPLC method

TABLE I
HPLC Separation Conditions for Diastereoisomeric Piperazine 2,5-Onthiones and Dithiones

	Analytical	Semipreparative	Preparative
Column	250 x 4 mm	500 x 8 mm	M 9 10/25 Whatman
Packing	Hypersil-silica 6 μm	LiChroprep silica 25-40 μm	Partisil-silica 10 μm
Detection	250, 254, 270 nm	250 nm	250 nm
Eluant:			
1. CH_2Cl_2-EtOAc	98:2	95:5 (v/v)	
2. EtOAc-MeOH-CH_2Cl_2		49.5:0.5:50	
3. Diisopropilaether-CH_2Cl_2-EtOAc		40:10:10	
4. Diisopropilaether-i-propanol-cyclohexane		5:1:60	5:1:80
Flow rate	0.8 mL min^{-1}	4 mL min^{-1}	2 mL min^{-1}
Instrument	Knauer-system	Knauer-system	Knauer-system
Sample	5 γ/μL	1 mg/200 μL	30 mg/500 μL
Solvent		Eluant + DMF	
Isolation		Fraction evaporation	
Purity checking	UV, CD, NMR	anal. HPLC	anal. HPLC

on Hypersil silica column with the same type of eluants ($\alpha = 1.2 - 1.6$). UV detection at 250, 254, 270, 279 or 281 nm (at the absorption maxima of the cyclic thioamides) was used in the HPLC measurements. The semipreparative normal phase separation of diastereoisomers was performed on Lichroprep and Partisil M-9 silica columns with eluants 1 - 4: [dichloromethane - ethylacetate - methanol or diisopropyl ether and i-propanol - cyclohexane - diisopropyl ether eluant mixtures] (Table I). The purity (usually > 94-95%) of the crystalline endproducts was checked by the above analytical HPLC system.

The diastereoisomers (in the order of elution: I and II, see Table II) were identified by UV, ^{1}H and ^{13}C NMR, CD and IR spectroscopy (14). Chromatographic conditions were optimized to achieve high resolution and baseline separation. Best results were obtained on Hypersil-silica columns. Fig 2. shows the typical chromatographic pattern for the separation of a diastereoisomeric cyclic dithioamide mixture.

The scale up of analytical separations was performed on preparative silica columns, silica packings of 63-125 μm and 48-63 μm were not efficient enough. The separation was improved by applying silica with 25-40 μm size. The best resolution was achieved on 10 μm silica column.

Due to the low tendency of shift reagents to form complexes with linear or cyclic thioamides, NMR spectroscopy could not be used for determining the L/D or L,L/D,D ratio of enantiomeric mixtures. Thus, comparative racemization studies were performed on

TABLE II
Diastereomeric Distribution of Cyclic Thiopeptides

Compound	Preparation method (Route)	Diastereomers k' I	Diastereomers k' II	α	Distribution % from HPLC	Distribution % from ^{1}H-NMR	Eluants
cyclo(Alat-Alat)	B	2.4	2.9	1.21	49.2:50.8	53:47	1
cyclo(Alat-Prot)*	B	1.0	1.5	1.50	49.1:50.9	41:59	3
cyclo(Alat-Prot)	A	2.2	2.5	1.14	49.0:51.0	45:35	4
crude product					39.1:60.9	40:60	
after preparative							
purification by HPLC					5.1:94.9 (II)**	3:97	
(one-step)					92.3:7.7 (I)	98:2	

*No separation in the eluent system 2.
**I = L, L + D, D
II = L, D + D, L
(I and II elution order of diastereoisomers; the compounds I and II were identified by CD and NMR)

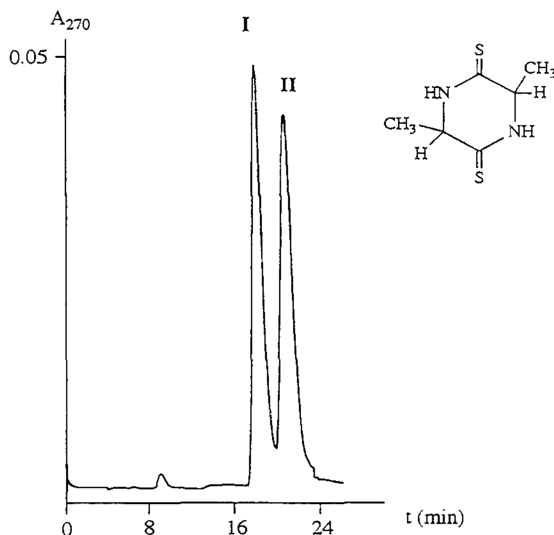


Figure 2. Analytical separation of diastereoisomeric cyclo-endothiopeptides. Col.: Hypersil-silica 250x4 mm; eluant: diisopropylaether-i-propanol-cyclohexane 5:1:60 v/v; flow rate: 0.80 ml min^{-1}; detection 270 nm; pressure 650 psi; sample: cyclo(L-Alat-L-Alat) PE 194 (I mixture of L,L and D,D enantiomers, II meso compound).

the amino acid mixtures obtained by acidic hydrolysis of linear and cyclic thiopeptides and on their (oxo)peptide precursors. The acidic hydrolysis using standard conditions (6M HCl, 48 hrs at 105°C in sealed glass tubes) was accompanied with complete dethionation. Isocratic reversed phase HPLC on ODS-Hypersil column with MeOH-CH_3CN or THF-NaOAc buffer (0.02M, pH 4) mobile phases and pre-column derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) were used to determine the ratio of D and L amino acids. Chromatographic data on Marfey's derivatives of amino acids contained in the models are summarized in Table III. The D/L amino acid ratio and rate of racemization of linear and cyclic thiopeptides and oxopeptides are given in Tables IV. and V.

We were able to monitor racemization for Ala and Pro residues and to determine the ratio of enantiomers by separation of the derivatized enantiomeric amino (oxo)acids ($\alpha = 1.5$-4.2, $R_s = 6.2$-12.9) (26-28). For *cyclo*(L-Alat-L-Alat), Fig. 3 shows the separation of D and L-Ala derivatives. The hydrolysis is also a potential source of racemization (27) so that this step was also carefully monitored.

As expected (29), the parent Z- or Boc-protected dipeptide esters and piperazine-2,5-diones prepared from them (see Table IV and V) were not found to suffer significant racemization during peptide synthesis, cyclization and acidic hydrolysis.

As reported earlier, the CD spectra of urethane protected endothiodipeptide esters of types Z(Boc)-Aaat^1-Aaa^2-OR and Z(Boc)-Aaa*t*-Gly-OR did not show dependence on the conditions (solvent,

TABLE III
Chromatographic Data of Amino Acid Marfey's Derivatives

Syst. amino acid	k' L	k' D	α	R_s	Eluent system
1. Ala	2.1	6.1	2.9	8.0	MeOH-CH_3CN-0.02M NaOAc buffer
	15.1				20:10:70
Marfey-OH*					
2. Ala	4.7	7.3	1.5	9.1	THF-0.02M NaOAc buffer
Gly	3.7				15:85
Marfey-OH*	34.0				
3. Pro	5.1	11.0	2.1	6.2	MeOH-CH_3CN-0.02M NaOAc buffer
					18:8:74
Marfey-OH*	22.0				
Gly	3.6				
Ala	6.0	14.2	2.1	7.0	
4. Val	3.0	12.5	4.2	12.9	MeOH-0.02M NaOAc buffer
					40:60
Marfey-OH*	5.1				
Gly	0.8				

System 1 for cyclo(Alat-Alat)
2 for cyclo(Alat-Gly)
3 for the others, e.g. cyclo(Alat-Pro), cyclo)Pro-Gly), etc.
4 for Val peptides

(*Marfey-OH is the hydrolyzed reagent)

HPLC. Column: ODS-Hypersil-6 (125x4 mm)
Flow rate: 1.1 mol min^{-1}
Detection: at 340 nm

TABLE IV
Racemization Data of diketopiperazines

Diketopiperazines	D/L amino acid ratio without* correction	with correction	Rate of racemization % without* correction	with** correction
cyclo(Ala-Ala)	6.6:93.4	1.8:98.2	13.2	3.6
cyclo(Ala-Gly)	2.69:97.4	0.05:99.95	5.2	0.1
cyclo(Pro-Ala)	7.4:92.6 (Pro)	0.5:99.5	14.8	1.0
	5.5:94.5 (Ala)	0.7:99.3	11.0	1.4
cyclo(Pro-Gly)	7.6:92.4	0.7:99.3	15.2	1.4

*Without correction, the values are together with the background racemization of single amino acid components.
**These data are in good correlation with ones of Morinobu (31).

temperature, reaction time etc.) of thionation (Scheme 1) (13, 14). Similarly, ^{1}H and ^{13}C NMR studies did not reveal significant amounts (> 1-2%) of diasteroisomeric impurities in samples of Z(Boc)-Aaat^1-Aaa^2-OR thiopeptides (13, 15). Most importantly, the oxopeptide Z-Val-Gly-OEt obtained from Z-Valt-Gly-OEt by dethiation with Ag_2O in a dioxane/water mixture (13), gives 99.7% L- and only 0.3% D-valine after acidic hydrolysis, while its precursor, Z-Valt-Gly-OEt, results in 11.5% D-valine.

A comparison of the above data leads to the conclusion that, contrary to (oxo)peptides, linear protected thiopeptides suffer significant racemization during acidic hydrolysis. In the isomeric endothiopeptides Z-(Boc)-Prot-Gly-OMe and Z(Boc)-Glyt-Pro-OMe, the chiral center preceding the thioamide bond is less sensitive to

TABLE V
Racemization Data of Linear Thiopeptides*

Linear endothiopeptides	D/L amino acid ratio	Rate of racemization** %
Z-Ala-Ala-OEt	1.0:99	2.0
Z-Alat-Ala-OEt	24.5:75.5	49.0
Z-Val-Gly-OEt	0.2:99.8	0.4
Z-Valt-Gly-OEt	11.5:88.5	23.0
"Z-Valt-Gly-OEt"***	0.3:99.7	0.6
Z-Prot-Pro-OMe	20.0:80.0	40.0
Z-Alat-Gly-OEt	1.5:98.5	3.0
Z-Glyt-Pro-OMe	23.0:77.0	46.0
Z-Prot-Gly-OMe	6.0:94.0	12.0
Boc-Prot-Gly-OMe	2.0:98.0	4.0
Boc-Glyt-Pro-OMe	28.0:72.0	56.0
Boc-Pro-Gly-OMe	1.1:98.9	2.2
Boc-Pro-Ala-OMe	0.5:99.95 (Pro)	0.1
	0.05:99.55 (Ala)	0.1
Boc-Prot-Ala-OMe	33.0:67.0 (Pro)	66.0
	28.0:72.0 (Ala)	56.0
Boc-Ala-Pro-OMe	0.05:99.25 (Pro)	0.1
	1.4:98.6 (Ala)	2.8

*The data are corrected always with the background racemization of single amino acid components.
**Racemization rate (%) = {100 × [2D:(D+L)]}, where D and L are peak areas of isomers on the chromatograms.
***After dethiation. The physical properties (m.p., optical rotation) support this data, too.

racemization than the chiral center succeeding it (Table V). The high racemization rates of both chiral residues in Boc-Prot Ala-OMe is in contradiction with this finding. A thioalanyl (-Alat-) residue appears to be more resistant to racemization during hydrolysis than a thioprolyl (Prot-) one.

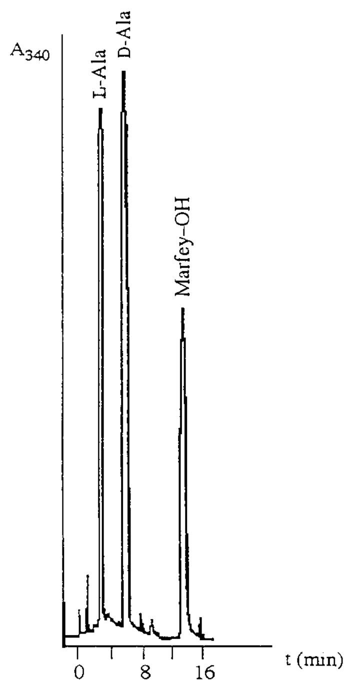


Figure 3. Separation of Marfey's derivatives. Col.: ODS-Hypersil-6, 125x4 mm; eluant: MeOH-CH_3CN-0,02M NaOAc buffer (pH 4); flow rate: 1.1 ml min^{-1}; detection: 340 nm; recorder speed: 15 cm/hr; pressure: 1350 psi; hydrolyzed sample: cyclo(L-Alat-L-Alat); derivatization: Marfey's reagent; sample volume: 10 µl.

The data listed in Tables II, IV-VII and the results of preliminary X-ray crystallographic, NMR and CD spectroscopic studes (14, 24) clearly indicate that piperazine-2,5-onthiones and 2,5-dithiones may undergo racemization not only during acidic hydrolysis but also in the course of their preparation, isolation or pu-

TABLE VI
Racemization Data of Cyclic Thiopeptides*

Cycloendothiopeptides	Preparation method (Route)	D/L amino acid ratio %	Rate of racemization**
cyclo(Alat-Ala)	A	23.0:77.0	46.0
cyclo(Alat-Alat)	B	49.5:50.5	99.0
cyclo(Prot-Prot)	B	29.8:70.2	59.6
cyclo(Alat-Gly)	A	44.0:56.0	88.0
cyclo(Alat-Glyt)*	A	0.0:100	0.0
cyclo(Prot-Pro)	A	18.0:82.0	36.0
cyclo(Prot-Gly)	A	46.7:53.3	93.4
cyclo(Glyt-Ala)	A	5.5:94.5	11.0
cyclo(Glyt-Pro)	A	30.0:70.0	60.0
cyclo(Prot-Ala)	A	46.0:54.0 (Pro)	92.0
		36.0:64.0 (Ala)	72.0
cyclo(Prot-Alat)	B	49.5:50.5 (Pro)	99.0

*Preparation at 20°C.

rification. The thionation of piperazine-2,5-diones and piperazine-2,5-onthiones is always accompanied with more or less extensive racemization, depending on the structure of the parent cyclic peptide or monothiopeptide and the conditions of thionation. It should be taken into account, that the formation of piperazine 2,5-diones (diketopiperazines) proceeds with some racemization (29). Their isomerization was studied theoretically and experimentally, too (21-23). The rate constant for racemization of *cyclo*-(L-Ala-Gly) (diketopiperazine) was only 2 times that of H-Gly-Ala-OH and 7 times the rate of H-Ala-Gly-OH; it racemized 20 times andH- Gly-Ala-OH

TABLE VII
Selected 400 MHz ^{1}H NMR Data on Piperazine Mono- and Dithiones[a]

	c(Alat-Glyt)[b]	c(Alat-Ala)[c]		c(Alat-Alat)[c]		c(Prot-Gly)[c]	c(Glyt)-Pro)[b]	c(Prot-Ala)[c]		c(Prot-Alat)[c]	
		LL(DD)	LD(DL)	LL(DD)	LD(DL)			LL(DD)	LD(DL	LL(DD)	LD(DL
Gly(t)α	4.28	-	-	-	-	3.72	4.07	-	-	-	-
						4.02	4.28				
Ala α	-	3.94	4.02	-		-	-	4.16	3.85	-	-
Ala β	-	1.34	1.32	-		-	-	1.32	1.33	-	-
Alat α	4.33	4.18	4.18	4.23	4.27	-	-	-	-	4.31	4.35
Alat β	1.58	1.47	1.44	1.53	1.51	-	-	-	-	1.54	1.43
Prot α	-	-	-	-	-	4.31	4.26	4.34	4.44	4.49	4.60
Prot β	-	-	-	-	-	2.12	2(3H)			2.35	2.10
						2.38		1.75-2.4	1.75-2.	2.42	2.50
Prot γ	-	-	-	-	-	1.8-1.9	2.29(1H)			1.9-2.0	1.9-2.0
Prot δ	-	-	-	-	-	3.44	3.63			3.60	3.65
							3.76	~3.4	~3.4	3.83	3.78
CONH	-	8.2	8.2	-	-	-	8.35	-	-		-
CSNH	10.24	10.48	10.46	10.64	10.61	n.a	-	10.52	10.60	10.76	10.81
$^{3}JCH_{\alpha,NH}$	n.a.	<1, or ~2~	5 or ~1	n.a.	n.a.	n.a	5.0+1.5	~2	4.1	<1	4.2

[a] δ(p.p.m) relative to internal TMS in DMSO-d_6 unless otherwise stated.
[b] Solvent DMSO d_6 + $CDCl_3$
[c] Solvent DMSO d_6

66 times faster than free alanine (23). Racemization may occur during treatment of peptides with LR in dry benzene, toluene or other solvents even at room temperature (Scheme 1). Considering the rate of racemization encountered during the acidic hydrolysis of linear thiopeptides, the data in Table V suggest that the preparation of piperazine-2,5-onthiones of type *cyclo*(Aaat^1-Aaa^2) (Aaa^2 $\neq$ Gly) goes practically *without racemization* if the thionation takes place prior to ring closure (Route A in Scheme 1). Glycine appears to enhance the tendency for racemization, especially in Aaa*t*-Gly position. (Note, that during acidic hydrolysis of protected endothiopeptides, (Table V) glycine more promotes racemization in Gly*t*-Aaa position.) Apparently, the racemization may also take place following the thionation reaction. Chemically and optically pure Z- or Boc-Pro*t*-Gly-OMe can be N-deprotected without racemization. The dipeptide ester H-Pro*t*-Gly-OMe undergoes cyclization, in hot alcohol, in the presence of 0.05 - 0.1 equiv. of a tertiary amine base. Surprisingly, the monothione *cyclo*(Pro*t*-Gly) was found to show practically no optical activity. Optically active (or partially active) product cannot be prepared even at extremely mild thionation and isolation conditions. Acidic hydrolysis, derivatization with Marfey's reagent and HPLC separation (28) proved that this monothione suffers complete racemization during the cyclization in alcohols or the subsequent chromatographic purification in aqueous buffers (mono- and dithio derivatives of piperazine-2,5-diones are not soluble in nonpolar solvents). Though in lower extent, glycine also promotes racemization of alanine in Aaa*t*-Gly po-

sition. Contrary to these, the isomeric monothio derivatives *cyclo*(Gly*t*-Pro) and *cyclo*(Gly*t*-Ala) can be obtained from (Z- or Boc)-Gly*t*-Pro-OMe or Gly*t*-Ala through the same steps in practically pure L-enantiomeric form. Similarly to *cyclo*(Ala*t*-Ala) and *cyclo*(Pro*t*-Pro), the source of racemization here is the acidic hydrolysis rather than the cyclization or purification of the product (cf. Table IV and V).

The racemization studies based on HPLC chromatographic separation of diasteroisomeric cyclic thiodipeptides and the Marfey's derivatives of their amino acid components lead to the following conclusions:

1. In the case of dipeptides of type Z(Boc)-Aaat^1-Aaa^2-OR (R = CH_3, C_2H_5) thionation and subsequent nonhydrolytic dethionation do not cause considerable racemization under standard conditions. The spontaneous cyclization of dipeptide esters (H-Aaat^1-Aaa^2-OR) also goes without significant racemization.

2. Acidic hydrolysis of thiopeptides results in partially racemized amino acids. The rate of racemization is much higher (25-45%) at endothiodipeptides (Z[Boc)-Aaat^1-Aaa^2-OR] than in the corresponding (oxo)peptides (< 2%) [Table IV-V.].

3. Thionation of piperazine-2,5-diones or piperazine-2,5-onthiones is always accompanied by racemization. The ratio of diastereoisomeric and/or enantiostereoisomeric products depends on the structure of the parent cyclic peptide or thiopeptide and the conditions of thionation. Due to steric reasons, thionation of *cyclo*(Pro-Pro) proceeds with partial double racemization (see Table VI). In the case of *cyclo*(Ala*t*-Alat) the meso-compound is also formed to-

gether with the racemic pair.

4. Cyclization of optically pure endothiodipeptide esters (H-Aaa*t*[1]-Aaa[2]-OR; Aaa[2] ≠ Gly) gives rise to piperazine-2,5-onthiones of type *cyclo*(Aaa*t*[1]-Aaa[2]). The comparison of chromatographic and NMR-based racemization data suggests that the cyclization and isolation of the products are free from racemization. partial racemization of the chiral amino acid residues occurs during the acidic hydrolysis. Glycine has a special positional effect on racemization: *cyclo*(Pro*t*-Gly) and *cyclo*(Ala*t*-Gly) suffer total racemization during ring-closure and isolation while *cyclo*(Gly*t*-Pro) and *cyclo*(Gly*t*-Ala) can be prepared in optically pure form.

5. The acidic hydrolysis of thiopeptides yields amino (oxo)acids. Thus, the pre-column derivatization method with Marfey's reagent followed by RP-HPLC can be applied for measurement of racemization also in the case of thiopeptides. The diastereoisomeric cyclic thiodipeptides formed in consequence of racemization can be separated efficiently by HPLC on silica columns.

Starting from our observations the theoretical basis of stereochemical behaviour of cyclothiopeptides is discussed elsewhere (30).

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A SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETECTING SULFONAMIDE RESIDUES IN SWINE SERUM AND TISSUES AFTER FLUORESCAMINE DERIVATIZATION

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ABSTRACT

A highly sensitive and rapid high-performance liquid chromatographic method for determining sulfonamides (sulfadiazine, sulfamethazine, sulfamonomethoxine, sulfamethoxazole and sulfadimethoxine) in swine serum and tissues is described. The sulfonamides were extracted from the samples, derivatized with fluorescamine, chromatographed on a Nova-Pak C_{18} column using acetonitrile-10 mM potassium phosphate (30:70, v/v) as the mobile phase and detected spectrofluororimetrically (excitation 390 nm, emission 475 nm). The retention times were 7.1 to 18.2 min and there was no interference from any co-extractives. The detection limit for each standard sulfonamide solution was 0.1 ng/ml and their calibration curves were linear between 1 and 100 ng/ml. In the presence of sulfadiazine as an internal standard, sulfonamide recovery from spiked serum, muscle, liver and kidney samples (10 ng/ml) was 94.0 ± 4.7 to 97.3 ± 5.9%, 58.5 ± 3.1 to 73.9 ± 5.7 %, 65.9 ± 7.1 to 86.9 ± 10.6% and 86.2 ± 4.0 to 92.8 ± 6.4% respectively.

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INTRODUCTION

Antimicrobial agents are given to animals in subtherapeutic concentrations for three reasons: (1) to prevent infectious diseases caused by bacteria or protozoa; (2) to decrease the amount of feed needed and (3) to increase the rate of weight gain [1]. Sulfonamides were the first chemotherapeutic agents used for the systematic control of bacterial diseases in livestock [2], as they had a broad spectrum of activity and were cheap [3]. However, the use of antimicrobial agents in animals that end up as food for human consumption results in the presence of illegal residues in meat (especially the liver and kidneys) [1]. A study by the National Center for Toxicological Research indicated that sulfamethazine may be a thyroid carcinogen [4].

Various authors have published procedures for determining different sulfonamides in animal fluids and tissues, most of which involve reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [2]. However, these methods require elaborate and time-consuming clean-up procedures or have low detection sensitivities. Japanese food safety laws have established a zero residual level for all antimicrobial agents in edible animal tissues [5]. Therefore, improved analytical procedures are needed to monitor them for sulfonamide, and other antibiotic, residues.

Fluorescamine was first reported to be a means of generating fluorescent derivatives of primary amino acids [6]. Sigel *et al.* [7] detected sulfadiazine after derivatization with fluorescamine solution using a thin-layer chromatographic (TLC) method and recently, several TLC methods [3, 8, 9] for analyzing sulfonamides at ppb levels in animal tissues using a fluorescamine solution for derivatization have been described. Although van Haaster *et al.* [10] developed a highly sensitive HPLC method for determining histamine and 3-methylhistamine in biological samples using fluorescamine as the derivatizing agent. So far, to our knowledge, no reports of methods for identifying sulfonamide residues in animal tissues using HPLC with fluorescamine derivatization have been published. The objective of this study was to develop a rapid and sensitive screening method for sulfonamide residues in swine serum and tissues using HPLC with fluorescamine as the derivatizing agent.

EXPERIMENTAL

Materials

Sulfadiazine (SDZ), sulfamethazine (SMT), sulfamonomethoxine (SMM) and sulfadimethoxine (SDM) were purchased from Sigma (St. Louis, MO, USA) and sulfamethoxazole (SMX) was obtained from Shionogi Pharmaceutical, Osaka, Japan. Acetonitrile, potassium dihydrogen phosphate (PDP) and N,N'-dimethylformamide were obtained from Wako Pure Chemical Industries, Tokyo, trichloroacetic acid (TCA, analytical reagent grade) was from Yoneyama Yakuhin Kogyo, Osaka, Japan, fluorescamine was obtained from Sigma and HPLC-grade water was produced in a Milli-Q purification system (Millipore, Milford, MA, USA). Stock sulfonamide solutions (1 mg/ml) were prepared by dissolving 10 mg each standard compound with 1 ml N,N'-dimethylformamide and diluting them to 10 ml with distilled water. The stock solutions were diluted to the desired concentrations (1, 5, 10, 50 and 100 ng/ml) with 0.01% TCA solution for calibration curve study. Sulfadiazine (10 ng/ml) was added to the assay solution as an internal standard.

Extraction procedure

Serum

A 0.1-ml aliquot of each sulfonamide solution (100 ng/ml) was added to 0.9 ml swine serum (spiked 10 ng/ml), then kept in the refrigerator (4°C) overnight to allow drug incorporation into the serum, after which 4 ml acetonitrile was added for extraction and deproteinization, the mixture was stirred with a vortex stirrer and centrifuged for 15 min at 1,000 g. The supernatant was evaporated to dryness under a stream of nitrogen gas using a 40°C water bath, the residue was dissolved in 0.05 ml water and mixed vigorously, 1 ml acetonitrile was added to the mixture, which was for centrifuged 15 min at 1,000 g and the resulting upper layer was evaporated to dryness. The residue was dissolved in 1.0 ml 0.01% (w/v) TCA solution containing 10 ng/ml sulfadiazine (internal standard) and shaken, 0.1 ml hexane was added to the solution, which was shaken again

and centrifuged at 1,000 g for 15 min, after which, a 0.5-ml aliquot of the clear layer was collected carefully with a Pasteur pippet and used for derivatization.

Tissues

Swine kidney, muscle and liver tissues (100-200 g) were cut into small pieces and homogenized in a blender. The ground tissues were stored at -30°C until analyzed, when 1.0 g was placed in a 10-ml centrifuge tube, 0.1 ml sulfonamide mixture (100 ng/ml) was added to produce 10 ng/ml spiked samples, which were kept in the refrigerator (4°C) overnight to allow drug incorporation into the tissues, after which, the extraction procedure was carried out as described for serum.

Derivatization

A 0.1-ml aliquot of freshly prepared fluorescamine solution in acetonitrile (1 mg/ml) was added to each tube containing 0.5-ml purified samples, which were shaken by hand 1 min, and a 50-μl aliquot of each derivatized sample was injected into the HPLC column.

HPLC analysis

The HPLC system comprised a Model 600E multisolvent delivery pump connected to a U6K injector (Waters Associates, Milford, MA) and a Hitachi F-1050 fluorescence spectrophotometric detector (Hitachi, Tokyo, Japan) operating at excitation (Ex) and emission (Em) wavelengths of 390 and 475 nm respectively. The separation procedure was performed using a Nova-Pack C_{18} column (prepacked, 10-μm particle size, 300 mm x 3.9 mm ID, Waters Associates), the chromatographic data system used was Chromatopac C-R6A (Shimadzu Seisaku, Kyoto, Japan), the mobile phase comprised acetonitrile-10 mM potassium dihydrogen phosphate (30:70, v/v) at room temperature, which was degassed using an ultrasonic bath and the flow-rate was 1.0 ml min^{-1}.

Calculation

A standard calibration curve (four replicates each) for each of the four sulfonamides of their peak-height (h) to that of the internal standard (IS) ratios against their concentrations (1, 5, 10, 50, 100 ng/ml) was plotted using the following equations: Y = aX + b and Y = h (of each sulfonamide)/h (IS); X = concentrations; a = slope; b = intercept. The recovery of each sulfonamide from each spiked sample was calculated by comparing its peak-height ratio with those of the standard control solutions under identical HPLC analytical conditions.

RESULTS and DISCUSSION

Linearity and stability

The linearity of thefluorescence intensity of the sulfonamide mixture solution was evaluated by analyzing a concentration range of 1 to 100 ng/ml of the mixture of four sulfonamides. The chromatogram of 0.5 ml standard sulfonamide mixture solution (1 ng/ml) containing 10 ng/ml sulfadiazine (internal standard) derivatized with 0.1 ml fluorescamine (1 mg/ml) is shown in Fig. 1. Each peak was symmetrical and the retention times of SDZ, SMT, SMM, SMX and SDM respectively were 7.1, 7,9, 9.1. 14.1 and 18.2 min.

The standard calibration curves (four replicates) for the four sulfonamides were linear with correlation coefficients in excess of 0.99 as follows: SMT: $Y=(0.1383 \pm 0.0033)X + (0.1315 \pm 0.0617)$, $r=0.9993 \pm 0.0005$; SMM: $Y=(0.1033 \pm 0.0037)X + (0.1202 \pm 0.0671)$, $r=0.9977 \pm 0.0025$; SMX: $Y=(0.0544 \pm 0.0032)X + (0.079 \pm 0.0546)$, $r=0.9987 \pm 0.0014$; SDM: $Y=(0.0458 \pm 0.0032)X + (0.0895 \pm 0.0504)$, $r=0.9974 \pm 0.0029$. In a standard solution, with a 50-μl injection sample, 0.1 ng/ml sulfonamides was the lowest concentration that could be detected. van Haaster *et al.* [10] detected 20 pg histamine and 3-methylhistamine on their column at a signal-to-noise ratio of 3:1, which was about the same sensitivity as our method. They also demonstrated that only 10% of the fluorescence intensity was lost over a period of 7 days. In this study, we

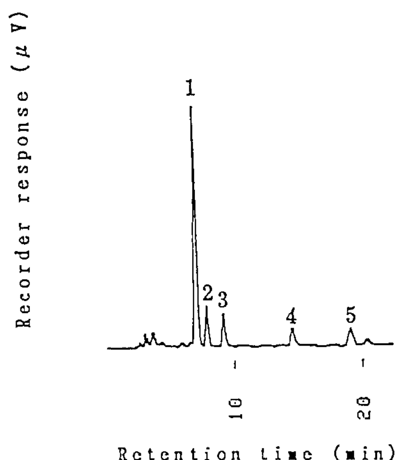

Figure 1. Chromatogram of the four standard sulfonamides (1: SDZ at 10 ng/ml, the internal standard, 2: SMT, 3: SMM, 4: SMX and 5: SDM with respective retention times of 7.1, 7.9, 9.1, 14.1 and 18.2 min) at 1 ng/ml derivatized with 0.1 ml fluorescamine solution (1 mg/ml).

analyzed 0.5 ml mixture of standard sulfonamides (10 ng/ml) solution on day 5 after derivatization with 0.1 ml fluorescamine (1 mg/ml), on day 5 and found the peak height was only about 10% (corresponding to about 1 ng/ml) of that obtained after derivatization for 1 min. Most of fluorophore activity appeared to have declined, which agrees with the results of Lai [11], who reported that the fluorescence intensity stayed constant for about 1 h, then diminished slowly thereafter van Poucke *et al.* [3] reported that after spraying with fluorescamine solution, the HPTLC plate should be scanned within 30 min. However, the fluorophore was found to be stable for up to 3 h in this study and its fluorescence intensity had halved 24 h after derivatization of a 10 ng/ml standard solution (0.5 ml) with 0.1 ml fluorescamine (1 mg/ml).

Recovery

The sulfonamide recoveries from swine 10 ng/ml spiked serum and tissue samples were determined using six replicates. The chromatograms of blank and spiked serum samples after acetonitrile extraction and fluorescamine derivatization are shown in Fig. 2. Those of the muscle, liver and kidney samples are shown in Figs. 3, 4 and 5 respectively. The recovery results for the four sulfonamides from swine spiked serum and tissue samples are presented in Table 1. The recoveries from muscle and liver were lower than those from serum and kidney. This may be due to an unknown substance that affected fluorophore formation or the sulfonamides may have bound to these tissues. Reimer and Suarez [8] demonstrated that low sulfonamide recovery from salmon muscle tissue appeared to be related to its relatively high cholesterol level.

Derivatization

Usually, o-phthalaldehyde is used as the derivatizing agent for determining sulfonamides with fluorescence detection. Morita *et al.* [12] analyzed sulfonamide residues in livestock products using HPLC with spectrofluorometric detection (Ex 285 nm, Em 445 nm). They used the o-phthalaldehyde as the derivatizing agent and the sample extracts had to be reacted with it for 30 min at 60°C. In this study, derivatization was quickly and easily carried out at room temperature for 1 min before injection into the HPLC column. Furthermore, Lai demonstrated that o-phthalaldehyde yielded background fluorescence several fold higher than fluorescamine [11]. Fluorescamine reacts with peptide primary amino groups almost instantaneously at room temperature in aqueous solution at pH 7.5-9 to form a fluorescent compound [11]. Initially, we used a solution of this pH for the derivatization reaction, but obtained a large interference peak that overlapped with the sulfonamides peaks. Eventually, we used 0.01% (w/v) TCA solution (pH 3.6) to dissolve the residues after drying under nitrogen gas and then derivatized them with fluorescamine for 1 min. The derivatized samples were eluted with a mobile phase (pH 5.3) of acetonitrile-10 mM PDP (30:70, v/v). These were found to be

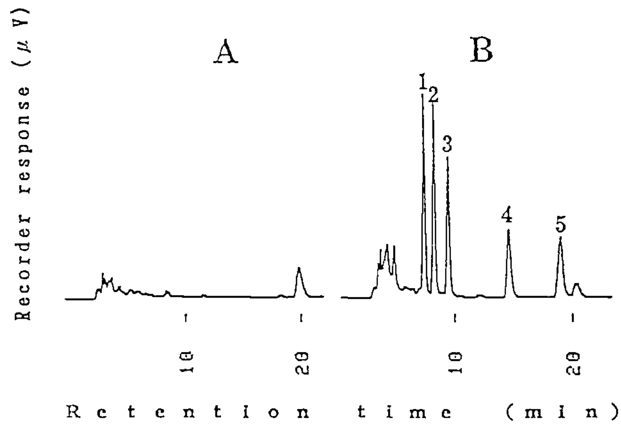

Figure 2. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank serum and (B) a serum sample spiked with standard sulfonamides (10 ng/ml) and the internal standard (10 ng/ml) sulfadiazine. The elution order is (1) SDZ, (2) SMT, (3) SMM, (4) SMX and (5) SDM with respective retention times of 7.1, 7.9, 9.1, 14.1 and 18.2 min.

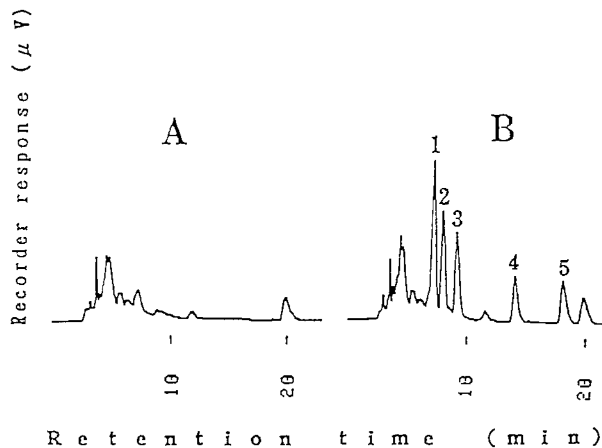

Figure 3. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked muscle samples. Spiking data and retention times as Figure 2.

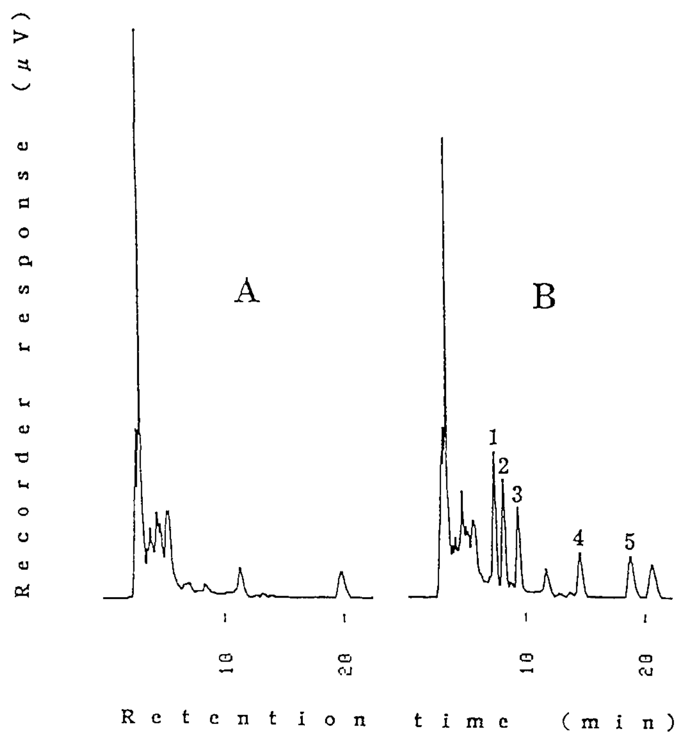

Figure 4. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked liver samples. Spiking data and retention times as Figure 2.

the best conditions for fluorophore formation and HPLC elution. Maybe owing to the HPLC system could change the circumstance for fluorophore formation.

Extraction

Usually, sulfonamides are extracted from solid samples, such as muscle and some tissues, by homogenizing the sample in an extraction solvent [3, 5, 8, 9, 12] and liquid samples, for example serum, milk and urine, are treated similarly by multiple extraction with organic solvents [2, 3, 12-14]. Both types of extract require additional clean-up

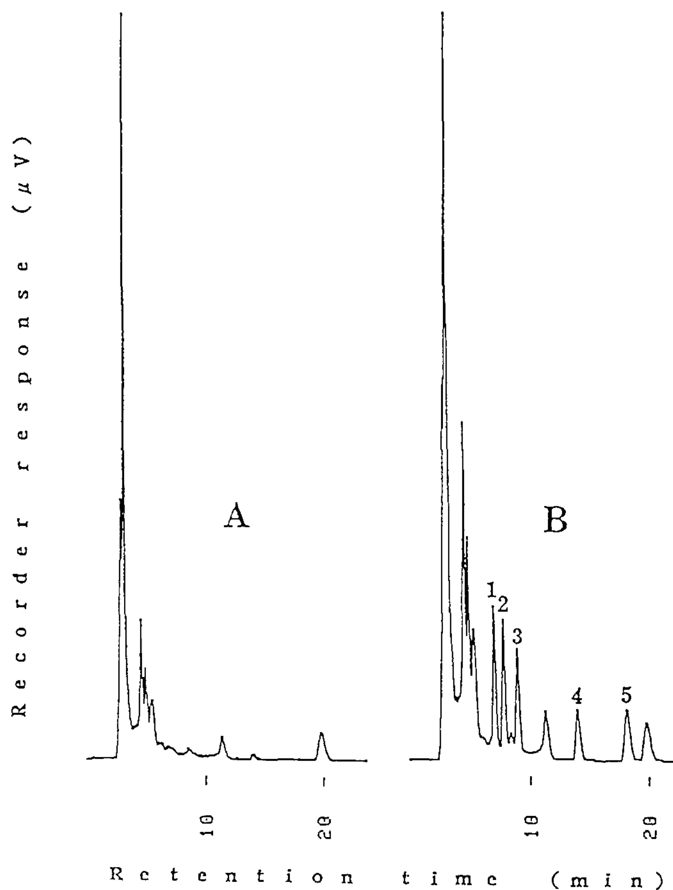

Figure 5. Chromatogram of fluorescamine-derivatized acetonitrile extracts from (A) blank and (B) spiked kidney samples. Spiking data and retention times as Figure 2.

steps and concentration using C_{18} packing material or another cartridge before they can be assayed [3, 5, 13] and these procedures are time-consuming. In this study, we developed a highly sensitive method for detecting sulfonamide residues at ppb levels. Only a small sample (1 ml serum or 1 g ground tissue) needs to be treated to monitor any residual sulfonamides and the extraction method is easy, using only 5 ml acetonitrile.

TABLE 1. RECOVERY RESULTS FOR THE FOUR SULFONAMIDES FROM SPIKED (10 ng/ml) SWINE SERUM AND TISSUES (n=6)

Drugs	serum	muscle	liver	kidney
SMT	97.0± 4.9	58.5± 3.1	65.9± 7.1	86.5± 4.3
SMM	94.1± 2.4	64.1± 2.7	73.5± 9.8	86.2± 4.0
SMX	97.3± 5.9	69.6± 3.2	83.1± 8.2	90.4± 5.0
SDM	94.0± 4.7	73.9± 5.7	86.9±10.6	92.8± 6.4

CONCLUSION

A simple, sensitive and rapid HPLC analytical method for determining sulfonamide residues in swine serum and tissues using fluorescamine as the derivatizing agent has been described. The advantages of this method over the others currently available are that a small sample only is needed, sulfonamide extraction is easy, derivatization with fluorescamine takes only 1 min at room temperature and at least four sulfonamides in a sample can be detected simultaneously. Our method may be useful for regulatory purposes for routine screening for some residual sulfonamides in animal edible tissues. However, further studies are necessary to evaluate the effectiveness of this system in vivo.

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ANALYSIS OF PRAZOSIN IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTION

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ABSTRACT

A high-performance liquid chromatographic procedure using fluorescence detection has been developed for the determination of prazosin in plasma. Propylhydroxybenzoate was used as the internal standard. The chromatography was performed using adsorbsphere phenyl column; the mobile phase consisted of 30:70% acetonitrile to 0.05 M phosphate buffer and was adjusted to pH 3.3-3.4 using phosphoric acid; a flow rate of 1.5 ml/min; and the effluent was monitored at excitation and emission wavelengths of 247 and 394 nm, respectively. The retention times for prazosin and the internal standard were 4.0 and 6.0 min., respectively. The intraday coefficients of variation (CV) ranged from 1.15 to 4.96% at three different concentrations and the interday CVs varied from 0.05 to 8.99%. The mean (± SD) absolute and relative recovery of prazosin were found to be 97.4±3.14 and 100.68±2.19, respectively. Stability tests showed that prazosin is stable for at least 2 weeks in plasma after freezing. The minimum detectable concentration of prazosin by this method was

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0.5 ng/ml. The sensitivity obtained should enable the use of this method in future bioequivalency and/or pharmacokinetic studies.

INTRODUCTION

Prazosin is a quinazoline derivative with a selective alpha$_1$-adrenoceptor blocking properties (1-3) that is widely used in the treatment of hypertension and heart failure (4-7). The usual initial dose of prazosin is 0.5 mg two or three times daily. The determination of plasma drug levels after such low doses required an assay capable of measuring levels below 1 ng/ml sample.

Numerous analytical methods have been described for assaying prazosin. These include spectroflurometry (8-11) and high-performance liquid chromatography (12-14). Generally, however, prazosin assays previously reported are time consuming involved double extraction steps and some of them suffer from a lack of sensitivity.

In this report a simple, rapid, sensitive, accurate and reproducible high-performance liquid chromatographic assay for the quantitative determination of prazosin in plasma is described. The method requires only 0.2 ml of plasma and involves a single extraction step, eliminating the tedious and time-consuming procedures required by the previously reported methods.

MATERIALS

Prazosin HCl was obtained from Sigma Chem. Co. (St. Louis, MO, USA) and propylhydroxybenzoate (internal standard) was obtained from E. Merck AG (Darmstadt, Germany). Acetonitrile and diethylether (BDH Chem. Ltd., Poole, U.K.) were HPLC grade. Sodium dihydrogen phosphate and disodium hydrogen phosphate and phosphoric acid (Riedel-De-Haen AG, Seelze, Hannover, Germany) were of analytical grade.

METHODS

Instruments

The following instruments were used:
A model LC-10AD solvent delivery pump (Shimadzu Corporation, Koyato, Japan), a model 470 fluorescence detector (Waters Associates, Milford, MA, U.S.A.), a model S/N 206003 chart recorder (Esterline Angus-Instrument Corp., Indianapolis, IN, U.S.A.), and a model 7010 Rheodyne injector (Rheodyne Inc., Catati, CA, U.S.A.). Chromatographic separation was performed using a stainless steel adsorbsphere phenyl column, 150 mm length x 4.6 mm i.d., 5 μm particles (Alltech).

Standard Solutions

Prazosin HCl (10 mg) and propylhydroxybenzoate (10 mg) were dissolved in methanol in two separate 100 ml

volumetric flasks to give standard stock solutions of 100 μg/ml.

Chromatographic Conditions

The mobile phase consisted of acetonitrile:0.05 M phosphate buffer (30:70% v/v) adjusted to pH 3.3-3.4 with phosphoric acid. The mobile phase was degassed by passing it through a 0.45 μm membrane filter (Millipore, Bedford, MA, U.S.A.) and pumped isocratically at a flow rate of 1.5 ml/minute, at ambient temperature. The effluent was monitored at excitation and emission wavelengths of 247 and 394 nm, respectively. The chart speed was 0.25 Cm/min.

Procedure

In a screw-capped glass centrifuge tube (10 ml), 0.2 ml plasma sample, 0.2 ml of 1 N NaOH, 12.5 μl of the internal standard solution and 7 ml diethylether were added. The mixture was shaken on a vortex mixer for 1 minute, and centrifuged for 2 min, at 3000 rpm. The ether layer was transferred into another glass centrifuge tube and evaporated to dryness. The residue was reconstituted in 0.4 ml of the mobile phase. An appropriate aliquot was then injected directly into the loop injector.

RESULTS AND DISCUSSION

The mobile phase reported herein (acetonitrile: 0.05 M phosphate buffer, 30:70% v/v, pH 3.3-3.4) was optimized for a rapid and interference-free chromatograms. The selected chromatographic conditions provided optimum resolution of prazosin and the internal standard. The retention times for prazosin and the internal standard were 4.0 and 6.0 min., respectively.

Figure 1 shows chromatograms from a drug-free blank plasma and plasma sample spiked with the drug and the internal standard.

Quantification

The quantification of the chromatogram was performed using peak-height ratios of the drug to the internal standard. Standard curves were constructed routinely from spiked plasma samples and mobile phase containing 0, 1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 ng/ml of prazosin. Four standard plots were obtained from plasma samples and six from the mobile phase. Least squares linear regression analysis of the calibration curves resulted in the following equations:

$$Y = -0.0210 + 0.1350\ X,\ r = 0.999 \text{ (Mobile phase)}$$

$$Y = 0.0070 + 0.1309\ X,\ r = 0.999 \text{ (Plasma)}$$

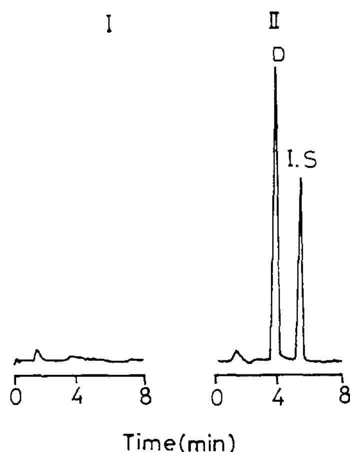

Figure 1 : Chromatograms of Blank Plasma (I) and Plasma Containing Prazosin and the Internal Standard (II)
Key : D : Prazosin
I.S. : Internal Standard.

Standard curves of prazosin in plasma and mobile phase were constructed on different days to determine the variability of the slopes and intercepts. The results showed little day-to-day variability of slopes and intercepts as well as good linearity ($r>0.99$) over the concentration range studied. The coefficients of variation for the slopes were 1.33% and 4.58% for the mobile phase and plasma, respectively.

Precision

The intraday precision was evaluated by replicate analysis of pooled plasma samples containing prazosin

Table 1 : Intraday and Interday Precision of Prazosin in Plasma.

Intraday*			Interday**		
Added Conc. (ng/ml)	Measured Conc. (ng/ml)	Bias*** %	Added Conc. (ng/ml)	Measured Conc. (ng/ml)	Bias*** %
7.5			7.5		
Mean	7.4	-1.3	Mean	7.52	0.2
S.D.	0.09		S.D.	0.01	
C.V. %	1.15		C.V.%	8.99	
15			15		
Mean	14.8	-1.33	Mean	15.1	0.66
S.D.	0.71		S.D.	0.01	
C.V.%	4.96		C.V.%	0.05	
30			30		
Mean	29.7	-0.01	Mean	31	3.33
S.D.	0.91		S.D.	0.72	
C.V.%	3.03		C.V.%	2.4	

* Mean values represent eight different plasma samples for each concentration.

** Interday reproducibility was determined From 8 different runs over 15-day period for the three concentrations.

*** Bias = 100 X (measured conc. - added conc.) / added conc.

at three different concentrations (low, medium and high). The intraday precision showed a coefficient of variation (CV) of 1.15% to 4.96% (Table 1). The inter-day precision was similarly evaluated over a 2-week period. The interday CVs ranged from 0.05% to 8.99% (Table 1).

Recovery

The absolute recovery of prazosin and the internal standard from plasma were assessed by comparing the peak height in plasma samples versus samples prepared in the mobile phase. The absolute recoveries ranged from 94% to 100.2% (Table 2). The relative recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma to the actual added concentrations. Eight comparisons at three different concentrations were made. As shown in Table 2, the mean relative recovery of prazosin from plasma ranged from 98.3% to 102.6%.

Stability

Stability studies of plasma spiked with prazosin (7.5, 15.0 and 30.0 ng/ml) were performed over a 15-day period (Table 3). Plasma samples were stored in the freezer at -20°C until the time of analysis. The results demonstrate that prazosin can be stored frozen in plasma for 2 weeks without degradation.

Sensitivity

The limit of quantitation for this method was found to be 0.5 ng/ml.

Table 2 : Absolute and Relative Recovery of Prazosin from Plasma* .

Conc. (ng/ml)	Mean Peak Heights (cm) Aqueous	Plasma	Absolute Recovery %	Relative Recovery % Mean ± SD	Range Relative Recovery %
7.5	1.98 ± 0.09	1.86 ± 0.05	94	101.15 ± 2.1	96 - 104.16
15	3.97 ± 0.07	3.90 ± 0.09	98	102.6 ± 5.1	95 - 108.1
30	7.86 ± 0.38	7.90 ± 0.09	100.2	98.3 ± 2.19	95.2 - 104.3
I.S. 2.5 μg/ml	1.8 ± 0.06	1.76 ± 0.08	97.8		

* Eight replicate analyses of each concentration .

Table 3 : Effect of Frozen Storage on Prazosin Stability in Plasma

Added Conc. (ng/ml)	Percent Recovery *			
	Days			
	0	**5**	**10**	**15**
7.5	98.0	101.0	96.0	98.0
15	95.0	98.0	101.0	101.0
30	102.0	101.0	98.0	103.0

* (Measured Conc. / (Added Conc.) X 100

Conclusion

The developed HPLC assay in this study has the sensitivity, rapidity, simplicity and reproducibility which makes it a potentially valuable tool in many applications such as drug level monitoring, drug-drug interactions, pharmacokinetic and bioequivalence studies.

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DETERMINATION OF WATER-SOLUBLE INORGANIC PHOSPHATES IN FRESH VEGETABLES BY ION CHROMATOGRAPHY

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ABSTRACT

An alternative Ion Chromatographic method has been developed for selective separation and quantitation of water-soluble inorganic phosphate in aqueous extracts of vegetables, based on the use of an anion-exchange polymethacrylate column, borate/gluconate as eluent and conductivity detection. The method shows a good detection limit as well as a high chromatographic resolution. It is also applicable to the detection of phosphates in fresh vegetables.

INTRODUCTION

Phosphate determination is of increasing importance in environmental matters regarding eutrophization phenomena in the biomedical fields, as well as in the food and beverage industry for effluent control.

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Recent studies show a considerable increase in daily phosphate intake (1,000-1,500 mg), due to changes in dietary habits (Recommended phosphate intake: 800 mg/day) (1). This increase leads to a P/Ca ratio higher than 2, with possible harmful effects on the mineral metabolism (osteoporosis, osteomalacia, decreased Mg absorption, ...) (2,3,4,5).

Generally, vegetables have high phosphorus contents due to inorganic fertilizers used in agriculture in order to obtain a higher yield and quality of these products (6) and for the purpose of antioxidant or protection from browning (7,8).

We considered the need for an analytical method which permits the study of residual inorganic phosphate in spanish vegetable produce in view of the increase in phosphate concentration levels in it, and the lack of data in the reference literature available.

Different techniques are routinely used for the determination of phosphate in foodstuffs. These include spectrophotometry (9,10), voltammetry (11) and flow injection analysis (12), but these methods suffer from various drawbacks, such as cumbersome sample preparation and long analysis time.

Ion Chromatography offers the opportunity to analyze ion species. Several applications of Ion Chromatography in analysis of phosphate in water (13), soil (14), fertilizers (15) and vegetables (16,17), have appeared in the literature.

In this work, we chose as working method an anion-exchange with conductivity detection and borate/gluconate as eluent to determine water-soluble inorganic phosphates in aqueous extracts of fresh vegetables.

MATERIAL AND METHOD

Samples

Analyzed samples were purchased from local food stores. Vegetables were kept refrigerated until assayed. Celery, chard, spinach and lettuce samples were analyzed within one day of purchase.

Reagents

All the reagents used were of analytic-reagent grade (Merck, D-6100 Damstadt, Germany). Organic solvents of high purity grade for HPLC (BDH, Poole, Dorset, UK). Ultrapure water with conductivity $< 1\ \mu S$ (DI water) was obtained from a Milli-Q (Millipore Corp., Bedford M.A. 01730, USA) four-bowl deionization system.

Phosphate stock standard solution was prepared at 1,000 ppm concentration by dissolving 0.1432 g of potassium dihydrogen phosphate ($K\ H_2\ PO_4$) per 100 ml in DI water. Working standard solutions were prepared daily by appropiate dilution of the stock solution with DI water.

Sodium Borate/gluconate concentrate solution was prepared with 16 g sodium gluconate, 18 g boric acid, 25 g sodium tetraborate decahydrate and 250 ml glycerin per 1,000 ml in DI water (concentrate may be stored for up to six months).

Sodium Borate/gluconate Eluent (conductivity 270 μS, pH 8.5) was prepared with 20 ml borate gluconate concentrate, 20 ml n-butanol and 120 ml acetonitrile to 1,000 ml. It was filtered through a 0.22 μm Durapore membrane (GVWP-Millipore), and degassed by ultrasonication before use.

Equipment

Chromatographic analysis was performed on an Ion Chromatography System ILC-1 (Waters Chromatography Division; Milford, MA, USA): Manual Injector with a 100 μl loop, Conductivity Detector (430), Programmable Solvent Delivery Module (590), Data Module Integrator (745). Precolumn Guard-Pak with IC-Pak anion inserts and 4.6 cm x 75 mm IC-Pak anion HR column (also Waters).

Method

250 g of each of the representative vegetable samples was cut into pieces and chopped in a domestic mincer. The marrow and onion samples had had their

skins peeled off previously. A subsample of 10.00 $\pm$ 0.1 g (5.00 $\pm$ 0.1 g in the case of samples with phosphate concentration higher than 1,000 μg/g, so that they were within the lineal range of this method) was homogenized with 100 ml of destilled water pre-heated to 70°C in a household mixer for 2 minutes. The mixture was heated on a boiling water bath for 15 minutes with repeated shaking, to denature and precipate the proteins.

The resulting extract solution was cooled, made up to 200 ml with water in a volumetric flask and filtered through Whatman n° 44 paper. Aliquots of 5 ml of the extracts were filtered through a 0.45 μm membrane filter (Millex HV - Millipore) to clarify them.

The purification was carried out applying this solution to a classic short body cartridge for solid phase extraction Sep-Pak C18 (Millipore - Waters), which was pretreated with 5 ml of methanol and 5 ml of water. The first 2 ml eluated were discarded and aliquots of 100 μl were injected into the chromatograph.

High Performance Ion Chromatography was carried out under the following conditions: conductivity detection; eluent, Sodium Borate/Gluconate pH 8.5 (conductivity 270 μS); flow rate, 0.9 ml/min.; chart speed, 0.5 cm/min; attenuation, 512; gain, 0.01.

RESULTS AND DISCUSSION

Ion Chromatography with conductivity detection is suitable for the determination of water-soluble inorganic phosphate (18, 19 y 20). Figure 1 shows the chromatogram of a standard solution containing 20 μg/ml of dihydrogen phosphate obtained under the chromatographic conditions previously described in the method.

Linearity and sensitivity of the method were calculated from a series of standard solutions from 1 to 50 μg/ml. Relationship between peak area and

FIGURE 1: Chromatogram of a standard solution of dihydrogen phosphate (20 μg/ml).
Conditions: Water IC-Pak HR anion column with Sodium Borate/Gluconate pH = 8.5 eluent; conductivity detection; flow rate: 0.9 ml/min; injection volume: 100 μl.

dihydrogen phosphate concentration was found to be linear over the 1-50 μg/ml concentration range. Equation of the least squares regression line was y = 0.23 - 0.26 x with a correlation coefficient of 0.999.

The limit of quantitation was 0.6 μg/ml allowing a signal noise ratio of 10. The limit of detection was estimated at 0.2 μg/ml to a signal noise ratio of 3.

The mean recoveries of standards in the 1-50 μg/ml range were 100.5 (σ = 4.6; n = 10).

Although Busman et al (14) suggest that the use of Ion Chromatography with conductivity detection in plant anion analysis was restricted by the presence of the organic compounds or by the high levels of salts, in the Ion Chromatography method developed in our laboratory and applied to water-soluble inorganic phosphate determination in aqueous extracts of several fresh vegetables, no interferences have been found from salts (chloride, nitrite, nitrate, sulphate) and organic compounds. Figure 2 shows the chromatogram of a standard solution of Cl^-, NO_2^-, NO_3^-, $H_2PO_4^-$, SO_4^{2-} anions, obtained under the chromatographic conditions described.

Figure 3 shows a typical chromatograms obtained from several samples of fresh vegetables (tomato, lettuce and marrow), where no chromatographic interferences can be observed.

Only in samples containing high levels of nitrate, the water-soluble inorganic phosphate couldn't be correctly quantified because resulting nitrate peak masks the phosphate peak. Figure 4 shows a chromatogram of a vegetable sample (chard) with a high nitrate concentration (4,500 μg/g), where it can appreciate that, though the nitrate concentration is very high, the phosphate peak is well resolved.

Furthermore Grunau et al (17) found that phosphate quantitation in vegetables aqueous extracts is less precise than other anions, due to the potential interference from proteins in plant extracts. To minimize the problem we have

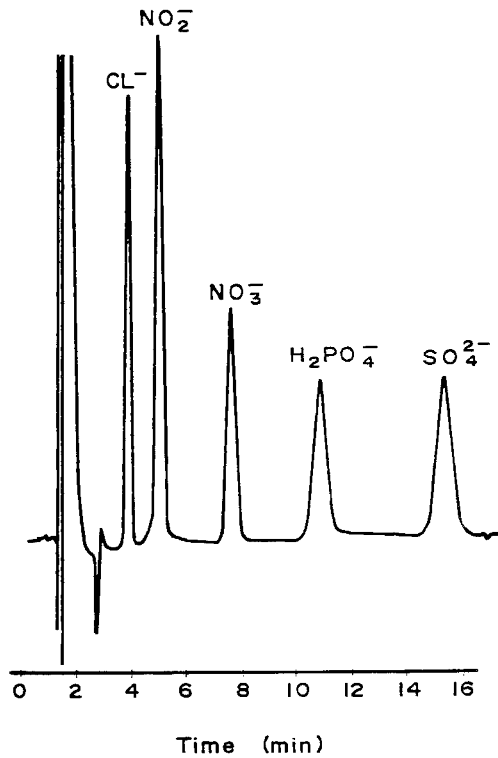

FIGURE 2: Chromatogram of a mixture of chloride, nitrite, nitrate, dihydrogen phosphate and sulphate standards (2, 4, 4, 6 and 4 respectly).
Conditions: Water IC-Pak HR anion column with Sodium Borate/Gluconate pH = 8.5 eluent; conductivity detection; flow rate: 0.9 ml/min; injection volume: 100 μl.

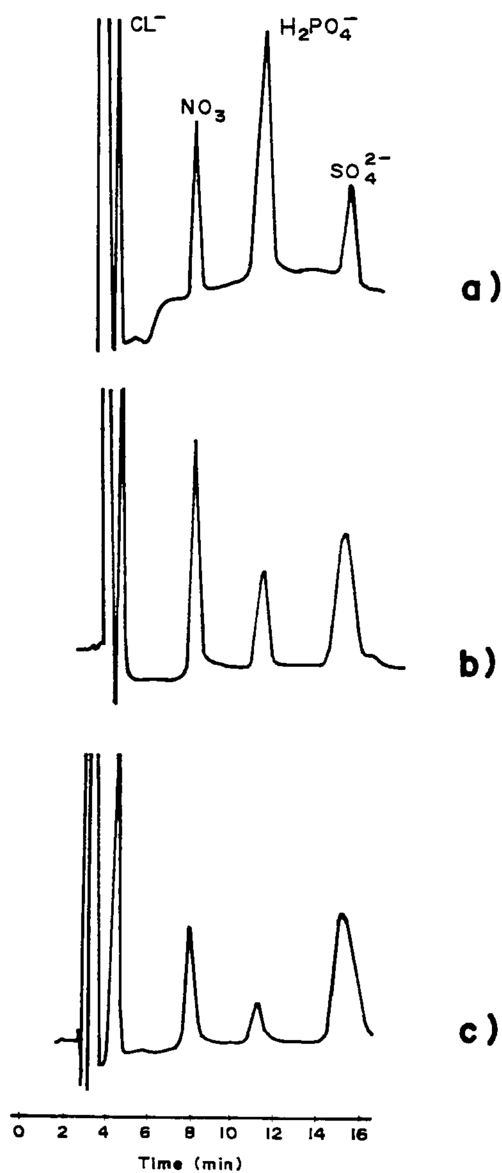

FIGURE 3: Typical chromatograms obtained from: tomato (a), lettuce (b) and marrow (c); using the proposed chromatographic method.

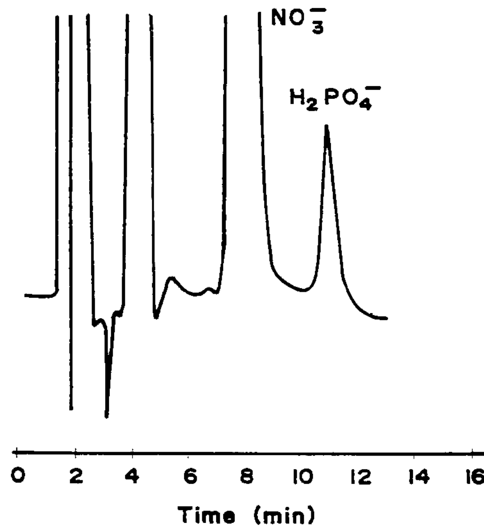

FIGURE 4: Chromatogram of a chard sample with high nitrate concentration (4,500 μg/g), obtained under the chromatographic conditions described.

chosen to desproteinize vegetable extracts routinely by boiling, primarily to avoid possible interferences and to prevent the column from clogging.

Recovery studies were performed on several fresh vegetable samples by adding known quantities of dihydrogen phosphate to the sample solution prior to the initial homogenization step. The results shown on Table 1, indicate that satisfactory recoveries were achieved for the samples tested.

A total of 76 different commercial fresh vegetable samples were analyzed: mushroom (8), celery (9), cauliflower (5), tomato (6), marrow (7), chard (8), onion (11), lettuce (10) and carrot (12). Water-soluble inorganic phosphate contents are shown on Table 2, where we can appreciate that all analyzed samples present high concentrations, the maximum amount reached being in the mushroom samples (mean value: 1,681.3 $\pm$ 393.5). In the other vegetable

TABLE 1

Percentage Recovery of Water-soluble Inorganic Phosphate from Fresh Vegetables after Extraction and IC Analysis

Food	Amount Added (μg/g)	Amount Recovered (μg/g) Mean ± SD	Recovery (%)	CV (%)
Chard	20	19.20 ± 1.69	97.20	8.80
(n=10)	40	41.18 ± 4.12	99.80	10.00
Lettuce	20	19.02 ± 1.58	97.70	8.30
(n=3)	40	40.93 ± 0.59	101.80	1.44
Spinach	20	18.01 ± 1.78	90.2	9.80
(n=3)	40	35.58 ± 1.22	89.2	3.40
Carrot	20	19.61 ± 0.49	98.2	2.49
(n=6)	40	39.99 ± 1.11	97.5	2.84
Marrow	20	20.02 ± 0.38	100.1	1.89
(n=4)	40	39.66 ± 0.58	99.1	1.46

samples analyzed, the mean values found ranged from 623.2 ± 297.4 (celery) to 357.5 ± 164.6 (carrot). Similar phosphate concentrations have been found in lettuce, spinach and tomato by Hertz et al (16).

To sum it up, several characteristics of the proposed method is a useful analytical technique for the determination of water-soluble inorganic phosphates: little sample preparation is required; it is not subject to organic, salts or protein interferences, and the precision of the technique is adequate for routine analysis, specially when the speed and cost-effectiveness of the method are considered.

TABLE 2

Water-soluble Inorganic Phosphate Contents in Commercial Samples

Sample (type)	n[a]	Mean (μg/g)	SD (μg/g)	Range (μg/g)
Mushroom	8	1681.3	393.5	1000-2174
Celery	9	623.2	297.4	222-1200
Cauliflower	5	612.2	246.6	228-925
Tomato	6	574.0	289.9	196-1060
Marrow	7	506.1	201.4	258-825
Chard	8	463.5	214.1	142-800
Onion	11	430.3	116.7	230-630
Lettuce	10	388.7	135.3	230-726
Carrot	12	357.5	164.6	156-608

[a] Duplicate determinations

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EVALUATION OF SULFOPROPYL ION-EXCHANGE MEMBRANE CARTRIDGES FOR ISOLATION OF PROTEINS FROM BOVINE WHEY

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ABSTRACT

Separated Cheddar cheese whey was microfiltered to remove residual fat, and adjusted to pH 3 prior to loading into two commercially available membrane cartridges. Minerals and non-protein nitrogen did not bind to the membranes. The mass of protein bound to the membranes increased as the loading volume of whey increased, while the percentage of protein isolated from the whey decreased. Not all of the protein bound to the membranes was eluted using pH 9 buffer. An economic analysis was used for comparison of the cartridges.

INTRODUCTION

Over 100 million pounds of whey protein concentrate (WPC) are used annually as a functional ingredient in bakery, dairy, cereal, beverage and other food

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products [1,2]. WPC is produced by ultrafiltration of whey. However, some undesirable properties of WPC limit its use, such as high lipid and lactose content, low foam formation, and poor foam stability [3]. These undesirable properties are nearly eliminated for whey protein isolate (WPI), which is made by adsorption of whey proteins onto ion-exchange (IEX) beads [3]. However, the cost of WPI is higher than that of WPC, due primarily to higher capital costs for building the IEX plant compared to the ultrafiltration plant [4]. In order to market WPI at a lower cost, either the process efficiency and throughput must be increased, or the capital cost must be decreased.

In commercial WPI manufacturing, whey proteins are adsorbed into IEX beads while whey and beads mix in a large stainless steel tank, followed by draining, washing, and elution of the adsorbed protein. The rate of protein isolation is limited by the rate at which equilibrium between the whey and the IEX beads is approached [5]. For large beads, this rate is slow because of lengthy diffusion times of protein into the IEX beads. Smaller beads decrease the diffusion time. However, these are not used in the commercial process because smaller beads also increase the time for liquid drainage from the tank.

IEX membranes are a new technology designed to overcome the limitations encountered in commercial IEX processes [5,6]. During adsorption, the whey passes through the micron-sized pores of the membrane and the proteins adsorb onto the IEX groups on the membrane surface. Diffusional limitations are negligible because the whey flows by convection through the fine pores of the membrane. Therefore, the IEX membrane process is expected to increase efficiency and throughput compared to the existing IEX processes. Similarities between IEX and ultrafiltration membrane equipment may permit existing WPC manufacturers to convert to WPI production without investing in a new plant. These factors may allow marketing WPI at a lower cost.

In this work, the feasibility of using IEX membranes for WPI production was investigated. Protein production rates, binding capacities, percentage of recovery, and processing parameters were evaluated for two membrane cartridges. The results of this work are useful in establishing the principles underlying the IEX membrane process, and in designing and operating new, more economical whey protein isolation and fractionation processes.

MATERIALS AND METHODS

Microfiltration

Separated (defatted) Cheddar cheese whey (pH 6.2), obtained from Associated Milk Producers, Inc. (Madison, WI) was cooled to 0°C. The whey was recirculated at 0-4°C through a hollow-fiber microfiltration membrane cartridge (model CFP-4-D-4, A/G Technology Corp., Needham, MA) containing polysulfone tubules with a pore size of 0.45 μm. The cartridge membrane area was 0.046 m^2. The pumping system consisted of a Masterflex® high-capacity pump drive (model 7549-30) and pump head (model 7019-00) (Cole Parmer Instrument Co., Chicago, IL). Raw whey (1500 mL) was recirculated until the volume was reduced by 50%. The permeate was adjusted to pH 3.0 using 0.375 M HCl and stored at 4°C. Four separate batches of whey were microfiltered, each having slightly different compositions.

Protein and Mineral Binding Study

Two commercially-available sulfopropyl strong-acid cation-exchange membrane cartridges were used to recover the whey proteins. One cartridge (Productiv® S, model PSC10-SP) was supplied by BPS Separations, Ltd. (Spennymoor, County Durham, U.K.). The unit consisted of a stack of 5 regenerated cellulose membranes. The stack had a bed height of 2 cm and a bed volume of 10 mL. Membrane pore size ranged from 50 to 300 μm. According to the manufacturer, the membrane binding capacity for lysozyme was 1 g.

The other cartridge (MemSep® 1010, model CISP 15H 01) was supplied by Millipore Corp. (Bedford, MA). The unit consisted of a stack of 72 SP regenerated cellulose membranes. The stack had a bed height of 1 cm, and a bed volume of 4.9 mL. The membranes had 85% void porosity, and 1.2 μm pore size. The capacity of the cartridge was reported by the manufacturer to be 2.3 meq, and the total binding capacity of lysozyme was 75-125 mg.

The protein recovery cycle consisted of equilibration, loading, washing, and elution. All steps were carried out at a constant flow rate using a Masterflex® drive (model 7520-25, Cole Parmer Instrument Co., Chicago, IL) and a FMI Lab

Pump Jr.® (model RHOCKC, Fluid Metering, Inc., Oyster Bay, NY). Equilibration consisted of pumping 10 bed volumes of the loading/washing buffer (L/W buffer) through the cartridge. Loading was accomplished by pumping whey solution at pH 3.0 through the IEX cartridge to adsorb proteins to the membranes. The L/W buffer was pumped through the unit to used to wash unbound materials from the membrane surface. The cycle was completed by pumping elution buffer (E buffer) through the unit to release bound proteins from the membranes.

When using the Productiv® S cartridge (PSC10), the L/W buffer was 0.1 M citric acid/sodium citrate pH 3.0, the E buffer was 0.2 M ammonium hydroxide/ammonium chloride pH 9.0, and the flow rate was 9.4 mL/min (one bed volume per min). When the MemSep® cartridge (CISP) was used, the L/W buffer was 0.02 M sodium acetate pH 3.0, the E buffer was 0.375 M Tris buffer pH 8.8, and the flow rate was 4.7 mL/min (one bed volume per min). The buffers used with the CISP were vacuum filtered with a 0.2 μm filter (model 66199, Gelman Sciences, Inc., Ann Arbor, MI) prior to use.

Cartridges were cleaned after the elution step. Eight bed volumes of 0.2 M NaOH solution at room temperature were pumped through the cartridge. The cartridge was then submerged in a 60°C water bath. After 1 hr, the cartridge was backflushed with another 8 bed volumes of the NaOH while still in the water bath. The cartridge was then removed from the water bath and backflushed with water at room temperature until the pH of the effluent was less then 8.0. Cleaning was completed by backflushing the cartridge with 16 bed volumes of 0.2 M HCl, followed by water until the pH of the effluent rose above 4. The cartridge was flushed with two bed volumes of L/W buffer prior to storage.

Cycle progress was monitored by a UV detector with a 10-μL-volume, 2-mm-lightpath flow cuvette (model 111, Gilson Medical Electronics, Inc., Middleton, WI) at a wavelength of 280 nm. The detector signal was recorded by a strip-chart recorder and a datalogger (model 50, Electronic Controls Design, Inc., Milwaukie, OR).

Seven separate cycles, each using a different loading volume of whey solution, were performed using the PSC10 to investigate the binding of total nitrogen (TN), non-protein nitrogen (NPN), protein and minerals as a function of the volume of whey solution loaded. In all these experiments, the volume of whey solution was loaded into the PSC10. Then the cartridge was flushed with L/W buffer until the detector signal returned to baseline. The detector signal from one experiment

using a loading volume of 250 mL was digitized and plotted as percent absorbance vs effluent volume. The effluent from the cartridge was collected continuously starting with loading of the whey solution, and ending with flushing with L/W buffer. It was then analyzed for TN, NPN and mineral composition. The elution peak was also collected and analyzed for mineral composition, and absorbance at 280 nm to determine total protein. Loading volumes of 10, 20, 30, 50, 100, 150, and 200 mL were used. One cycle using a loading volume of 50 mL was repeated at a flow rate of 4.7 mL/min. The procedure was repeated using the same PSC10 cartridge and loading volumes of 10, 20, 30, 40, 60, 150, and 250 mL. Four of these loading volumes were duplicates of the first procedure, and three were new. Only elution peaks were collected and analyzed in the later procedure.

Next, the amount of protein and minerals recovered in the elution peak as a function of the volume of whey loaded was determined for the CISP cartridge. Nine separate cycles were performed using loading volumes of 5, 10, 15, 20, 30, 50, 75, 100, and 125 mL of whey solution. This procedure was later duplicated using a different lot number CISP cartridge. The detector signal from the experiment using a loading volume of 75 mL was digitized and plotted as percent absorbance vs effluent volume. In each of these experiments, the elution peak was collected and analyzed for protein and mineral composition.

Protein and Mineral Analyses

The NPN content of raw whey, whey solution, and effluents was determined as follows: proteins were precipitated by adding 5 mL of 48 % (w/w) trichloroacetic acid (TCA) solution to 15 mL of sample. Then the Kjeldahl TN content in the filtrate from TCA precipitation, and in the untreated sample were determined by the University of Wisconsin Soil and Plant Analysis Laboratory. The total protein content of each sample was calculated as 6.38x(TN-NPN). The same laboratory also determined the mineral content of the samples using atomic absorption spectroscopy.

In order to convert the absorbance at 280 nm for each elution peak to total protein concentration, a conversion factor was determined by dialyzing six elution peak samples prior to measurement of total protein content. A 25-mL portion of

each sample was dialyzed against 0.10 M phosphate buffer, pH 8.8, using 2000 molecular weight cut-off dialysis tubing (Spectra/Por® 6, Spectrum, Houston, TX). The TN content of the dialyzed sample was determined, and 1 part dialyzed sample was diluted with 9 parts of 0.375 M Tris buffer pH 8.8 before measurement of the absorbance at 280 nm. Based on the absorbance of each dialyzed and diluted elution peak sample, and the total protein content calculated from the TN content, a conversion factor of 0.7 mg protein/mL/a.u./cm was used to convert the absorbances of the elution peak samples to protein concentration.

RESULTS AND DISCUSSION

Microfiltration

Loading raw whey directly onto the CISP cartridge created a rapid pressure increase, probably due to micron-sized particulates such as lipoproteins [7]. For this reason, the raw whey was microfiltered using a 0.45-μm polysulfone hollow-fiber membrane prior to loading into the cartridges. The turbidity of the permeate from microfiltration was less than that of the raw whey, probably due to a reduction in the lipoprotein content.

Raw whey and whey permeate (the whey solutions) were analyzed for protein, NPN and mineral content to determine if these components were reduced in concentration by microfiltration. The results of the microfiltration of four separate raw whey samples are contained in Table 1. The dilution factor which occurred on adjustment of the pH was used in calculating the original permeate composition. Based on these data, 70% of the total protein in the raw whey was recovered in the microfiltration permeate. This value is only moderately lower than the 79-80% recovery for a metallic microfiltration membrane at 50°C [8]. The permeate contained 84% of the NPN in the raw whey. Microfiltration did not significantly reduce the mineral content of the whey. The 30% reduction in total protein content of the whey due to microfiltration may have resulted from removing relatively more bovine immunoglobulin G and bovine serum albumin than α-lactalbumin and β-lactoglobulin from the raw whey [8].

TABLE 1

Effect of Microfiltration on Whey Composition

	Raw	Permeate
Volume (mL)	$1520 \pm 30^{\dagger}$	750 ± 20
NPN (mg/mL)	0.5 ± 0.2	0.42 ± 0.07
Protein (TN-NPN)x6.38 (mg/mL)	6 ± 1	4.2 ± 0.4
K (mg/mL)	1.3 ± 0.2	1.18 ± 0.05
Na (mg/mL)	0.37 ± 0.02	0.37 ± 0.02
Ca (mg/mL)	0.42 ± 0.09	0.38 ± 0.01
Mg (mg/mL)	0.07 ± 0.01	0.061 ± 0.006

†mean ± st. dev., n = 4

Optimum pH for Protein Adsorption

In the commercial stirred-tank process, using cationic silica-based resins with attached sulfonic groups (—SO_3^-), the whey is adjusted to pH 4.5 or lower to maximize protein adsorption [2,9]. For the IEX membrane process, the highest protein adsorption occurred at pH 3.0. A decrease in protein binding was observed when solutions of pH 3.5, 4.0 and 4.5 were used (data not shown). These findings agree well with those for sulfopropyl ion-exchangers which adsorb whey proteins efficiently from pH 1.5 to 3.5, with an optimum pH of 3.0 [4,10].

Composition of the Effluent when Loading Different Volumes of Whey Solution

Table 2 contains the amount of whey components in the loaded whey solution and in the effluent solution from the PSC10 cartridge when loading different volumes. These data were used to determine the degree of binding of each compound to the membrane. For all seven whey loading volumes, the amounts of NPN, potassium, calcium, and magnesium contained in the whey solution loaded into the cartridge nearly equaled the amounts in the effluent solution. Thus, none of these compounds bound to the membrane cartridge.

The amount of sodium loaded into the membrane cartridge came from both the whey solution and the L/W buffer. The amount of sodium loaded into the cartridge nearly equaled the amount in the effluent solution. Thus, sodium was not significantly retained by the membrane cartridge.

Conversely, amounts of TN and total protein in the whey solution loaded into the cartridge were substantially greater than amounts in the effluent solution. As expected, protein strongly bound to the membrane cartridge.

For pure solutions of single proteins, all the protein loaded into an IEX membrane binds until the saturation capacity is reached, at which point no more protein is retained [6]. As a result, prior to saturation, the total amount of protein bound to the cartridge should increase linearly with increasing loading volume.

In Fig. 1, however, the total amount of protein bound to the cartridge P (mg) generally increased non-linearly with increasing loading volume V (mL). The protein binding data (solid triangles) were fit by least-squares regression to the equation:

TABLE 2

Contents of Effluent vs Loading Volume of Whey Solution Using a PSC10 Cartridge

Loading volume (mL)	Effluent volume (mL)	TN (mg)		NPN (mg)		Protein (TN-NPN)x6.38 (mg)		K (mg)		Ca (mg)		Mg (mg)		Na (mg)	
		L[†]	E	L	E	L	E	L	E	L	E	L	E	L	E
10	73	10	1	3	3	42	-15	10	13	3	3	1	1	79	73
20	58	19	5	6	5	83	4	21	24	7	6	1	1	52	52
30	101	29	7	10	2	125	32	31	36	10	10	2	2	95	82
50	107	49	23	16	19	208	24	51	59	17	16	3	3	84	76
100	162	97	45	32	34	416	72	103	97	34	30	6	5	106	120
150	210	146	95	48	49	624	293	154	170	52	47	9	9	121	123
200	260	194	131	64	74	831	361	206	214	69	71	12	12	137	135
50*	81	49	19	16	17	208	15	51	58	17	18	3	3	53	58

[†]L = mg loaded into the membrane cartridge, E = mg in effluent solution

*flow rate = 4.7 mL/min

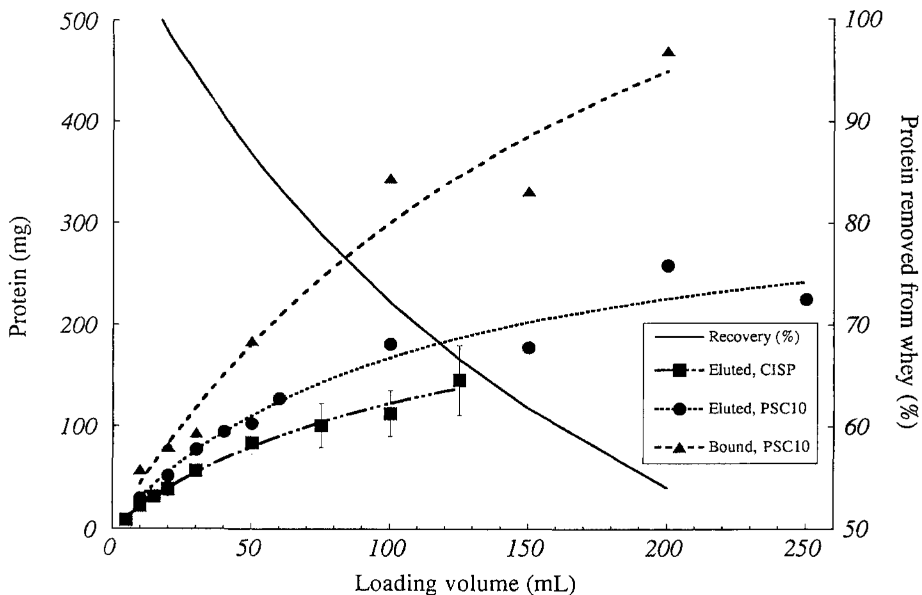

FIGURE 1. Protein bound to the PSC10, and protein in the elution peaks for the PSC10 and CISP vs the volume of whey solution loaded. Also plotted is the percentage of whey protein loaded which bound to the PSC10. Error bars indicate ± st. dev., n = 2.

$$P = p\frac{(V)}{(V + v)}$$

resulting in p = 890 mg, and v = 197 mL. The parameter p is the maximum amount of protein that would bind to the cartridge at infinite loading volume. The parameter v is the loading volume for binding of 50% of p.

The non-linear behavior of protein bound vs. volume loaded may have resulted from competitive adsorption for membrane binding sites between the individual proteins in whey. Competitive adsorption occurs for adsorption of whey proteins to cation-exchange membranes [6], and for whey protein adsorption to columns packed with Spherosil S cation-exchange beads [10].

Using the non-linear fit from Eq. (1), the greatest amount of protein which bound to the membrane was 450 mg for a loading volume of 200 mL. However,

as the loading volume increased, a smaller percentage of the protein in the whey solution loaded bound to the membrane cartridge. Using Eq. (1), the percent protein recovery was calculated and plotted in Fig. 1. Protein recovery was 87% or more for loading volumes of 50 mL or less, but it decreased to 54% for the highest loading volume of 200 mL. Therefore, there was a balance between operating conditions at one extreme where the percent retention is highest, and the other extreme where the amount of protein bound to the membrane was highest.

Composition of the Elution Peaks when Loading Different Volumes of Whey Solution

In these experiments, the amounts of protein and minerals in the elution peaks were determined after loading 10 to 250 mL of whey solution into the PSC10 cartridge, and 5 to 125 mL of whey solution into the CISP cartridge. This range of loading volumes for the PSC10 and the CISP was from 1 to 25 bed volumes. These loading volumes contained from less than 5% to over 100% of the amount of protein able to bind to the cartridges as reported by the manufacturers.

Tables 3 and 4 contain the protein concentration and the amount of minerals in the elution peaks for the PSC10 and CISP cartridges, respectively. The amounts of potassium, calcium, and magnesium in the elution peaks were negligible. These results agree with the results of Table 2, where these minerals were found not to bind to the cartridge. The amounts of sodium in the elution peaks were relatively small and constant, but not negligible. The average amount of sodium in the elution peaks was 25 mg for the PSC10, and 8 mg for the CISP. As mentioned before, sodium was not retained by the PSC10 cartridge (Table 2). However, before loading, the membrane cartridge was probably in the sodium form due to the equilibration step with L/W buffer. The sodium in the elution peak may have resulted from displacement of this sodium by the elution buffer.

The total amount of protein in the elution peak generally increased with increasing loading volume for both the PSC10 and CISP (Fig. 1). The data were fit to Eq. (1) resulting in p = 345 mg, and v = 106 mL for the PSC10 (solid circles), and p = 265 mg, and v = 117 mL for the CISP (solid squares). Using Eq. (1), the greatest amount of protein eluted from the PSC10 was 240 mg for a loading volume of 250 mL (25 bed volumes). For the CISP, 140 mg was eluted

TABLE 3

Contents of Elution Peak vs Loading Volume of Whey Solution Using a PSC10 Cartridge

Loading volume (mL)	Eluant volume (mL)	Protein (mg/mL)	K (mg)	Ca (mg)	Mg (mg)	Na (mg)
10	64 ± 4†	0.46 ± 0.02	0.05 ± 0.06	0.5 ± 0.2	0.1 ± 0.0	29 ± 7
20	54 ± 0	0.95 ± 0.01	0.13 ± 0.04	0.8 ± 0.2	0.15 ± 0.07	27 ± 5
30	77 ± 6	1.00 ± 0.02	0.15 ± 0.06	0.6 ± 0.0	0.1 ± 0.0	24 ± 1
40	79	1.20	0.1	0.3	0.7	23
50	67	1.53	0.2	0.9	0.2	21
60	67	1.90	0.2	0.7	0.1	26
100	94	1.92	0.1	0.3	0.1	26
150	93 ± 4	1.91 ± 0.07	0.4 ± 0.5	0.3 ± 0.3	0.1 ± 0.0	27 ± 4
200	125	2.07	0.4	0.5	0.1	25
250	88	2.56	0.2	0.4	0.1	21
50*	75	1.78	0.2	1.0	0.2	30

†mean ± st. dev., n=2

*flow rate = 4.7 mL/min

TABLE 4
Contents of Elution Peak vs Loading Volume of Whey Solution Using a CISP Cartridge

Loading volume (mL)	Eluant volume (mL)	Protein (mg/mL)	K (mg)	Ca (mg)	Mg (mg)	Na (mg)
5	14 ± 4†	0.6 ± 0.1	0.6 ± 0.7	0.01 ± 0.01	0.006 ± 0.006	8 ± 4
10	35 ± 4	0.6 ± 0.2	4 ± 6	1 ± 1	0.2 ± 0.3	8.60 ± 0.02
15	30 ± 3	1.0 ± 0.1	4 ± 5	1 ± 1	0.2 ± 0.3	7.28 ± 0.08
20	39 ± 5	1.0 ± 0.3	2 ± 2	2 ± 2	0.3 ± 0.2	7.16 ± 0.02
30	42 ± 4	1.4 ± 0.1	1 ± 1	2 ± 2	0.25 ± 0.07	8 ± 1
50	54.3 ± 0.4	1.5 ± 0.2	2 ± 2	2 ± 1	0.34 ± 0.08	7.6 ± 0.4
75	73 ± 9	1.4 ± 0.1	1.0 ± 0.7	2 ± 1	0.27 ± 0.03	9.5 ± 0.8
100	81 ± 2	1.4 ± 0.3	0.8 ± 0.4	3 ± 1	0.38 ± 0.05	8.0 ± 0.9
125	116 ± 7	1.3 ± 0.4	1.3 ± 0.9	2.8 ± 0.4	0.4 ± 0.1	9 ± 1

†mean ± st. dev., n=2

for a loading volume of 125 mL (25 bed volumes). However, the membrane volume of the PSC10 was about twice that of the CISP. The cartridges had similar capacities when compared on a basis of mg protein per mL membrane. The greatest amount of protein eluted from the CISP was 29 mg per mL of membrane, and from the PSC10 was 24 mg per mL of membrane.

Increasing the volume of whey solution loaded decreased the ratio of minerals-to-protein in the elution peak. The mineral-to-protein ratios of the elution peaks were as low as 0.1 mg/mg for the PSC10 using a 250 mL loading volume (Table 3), and 0.09 mg/mg for the CISP and a 125 mL loading volume (Table 4). In contrast, microfiltered whey permeate contained 0.47 mg minerals per mg protein (Table 1). Therefore, IEX membranes may be used to produce whey protein with a low mineral content. This is preferred in the production of infant formula and dietetic food products, because high mineral content can affect both the flavor and nutritional value of these products [9,11].

Breakthrough Curves from the PSC10 and CISP

Not all the protein which bound to the PSC10 was desorbed into the elution peak. Based on the fits of Eq. (1) to the data in Fig. 1, and Tables 2 and 3, 69% of the protein retained by the PSC10 was desorbed into the elution peak for a loading volume of 10 mL. This value dropped to 50% for a loading volume of 200 mL. Much of the protein which did not desorb into the elution peak can be accounted for by examining the digitized breakthrough curves.

In a typical breakthrough curve for the PSC10 (Fig. 2), the effluent absorbance rose rapidly to the feed solution absorbance, and slowly returned to baseline during washing. The elution peak emerged over an effluent volume of 142 mL. Only the first 88 mL of the elution peak were collected in order to avoid dilution of the peak due to tailing. Thus, a small amount of the protein desorbed from the membrane was lost in the 54 mL of the elution peak which was not collected. Finally, the small peak that emerged during cleaning with 0.2 M NaOH contained protein which was not desorbed by the elution buffer. The sum of these losses may account for the difference in the amount of protein collected in the elution peak (Table 3) compared to the amount bound to the cartridge after loading and washing (Table 2).

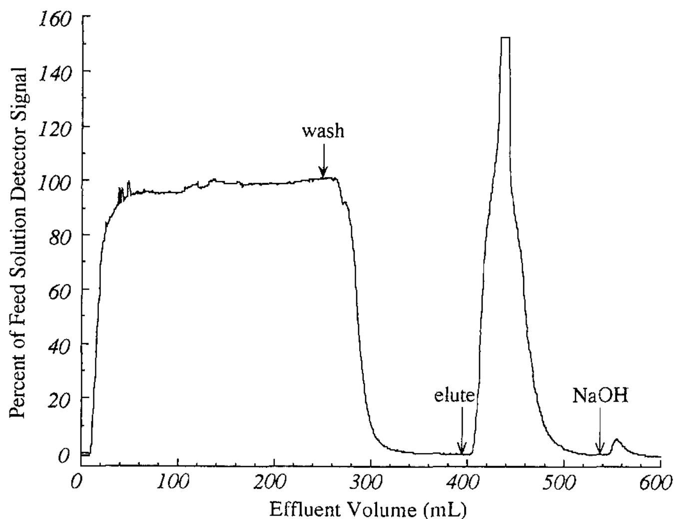

FIGURE 2. Breakthrough curve from the UV detector for the PSC10 cartridge. An aliquot of 250 mL whey solution pH 3 was loaded into the cartridge. The cartridge was washed using 145 mL 0.1 M citric acid/sodium citrate pH 3, and eluted using 142 mL 0.2 M ammonium hydroxide/ammonium chloride pH 9. The flow rate was 9.4 mL/min.

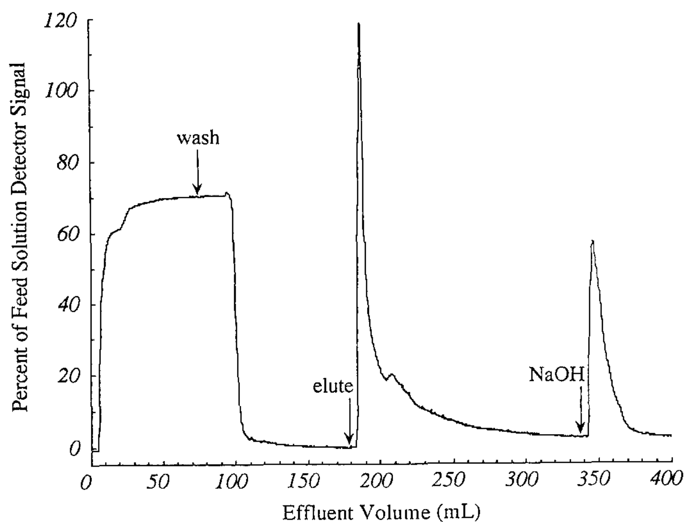

FIGURE 3. Breakthrough curve from the UV detector for the CISP cartridge. An aliquot of 75 mL whey solution pH 3 was loaded into the cartridge. The cartridge was washed using 103 mL 0.02 M sodium acetate pH 3, and eluted using 154 mL 0.375 M Tris pH 8.8. The flow rate was 4.7 mL/min.

In the breakthrough curve for the CISP (Fig. 3), the effluent absorbance reached a plateau at 70% of the feed solution absorbance, and slowly returned to baseline during washing. The elution peak emerged over an effluent volume of 154 mL. Because the elution peak had a long tail, only approximately the first 73 mL were collected. Thus, a significant amount of the desorbed protein was lost in the 81 mL of the elution peak which was not collected. Finally, the peak that emerged during cleaning with 0.2 M NaOH contained a large amount of protein which was not desorbed by the elution buffer. Based on the breakthrough curves in Figs. 2 and 3, the sum of the losses of protein for the CISP appears to be greater than those for the PSC10.

The failure of the effluent absorbance from the CISP to reach the feed solution absorbance in Fig. 3 could have resulted from removal of trace amounts of sub-micron particulates from the feed solution. The effluent from the CISP was visibly less turbid than the feed solution. The effluent absorbance from the PSC10 did reach the feed solution absorbance in Fig. 2. The larger pore size of the PSC10 (50 to 300 μm) compared to the CISP (1.2 μm) probably resulted in negligible removal of sub-micron particulates from the feed solution by the PSC10.

Effect of Flow Rate on Protein Recovery from the PSC10

In order to determine if the results depended on flow rate, the cycle using a loading volume of 50 mL was repeated at 4.7 mL/min, one half the standard value. From Table 2, none of the NPN or minerals bound to the membrane cartridge, in agreement with the results from the cycle using the standard flow rate. Protein binding was 5% higher compared to the cycle using the standard flow rate. However, this difference was within the normal range of variation of the results and may not have been significant. From Table 3, the amounts of minerals in the elution peak were nearly identical to those for the higher flow rate, in agreement with all previous results on mineral retention. The amount of protein in the elution peak for the lower flow rate was 30% higher than for the cycle using the standard flow rate. However, this increase in amount of protein may have resulted partly from the larger volume of the elution peak collected at the lower flow rate (Table 3). Three conclusions can be made: (1) NPN and mineral binding did not depend on flow rate, (2) the NPN and mineral binding data were highly reproducible, (3) protein binding did not depend strongly on flow rate.

Economic Considerations

An economic analysis of the two membrane cartridges favors the PSC10. The selling price of the PSC10 is 1.2 % of the selling price of the CISP for laboratory-scale cartridges on a per mL of membrane basis. However, the protein binding capacities of the cartridges are similar on a per mL of membrane basis.

Microfiltration of the whey substantially adds to the overall processing costs because it reduces the protein content of the whey by 30%, and it involves an extra processing step. Microfiltration was required for the CISP because direct loading of whey created a rapid increase in pressure. The larger pore size of the PSC10 (50 to 300 μm) compared to the CISP (1.2 μm) may allow for direct loading of whey without microfiltration, and it reduces the pressure drop at a given flow rate. The pressure drop was 10 psi at 9.4 mL/min for the PSC10, and 30 psi at 4.9 mL/min for the CISP. Consequently, whey processing costs for the PSC10 may be lower than for the CISP.

Because of low pressure drops at high flow rates, the cycling times for IEX membranes would be much shorter than those for commercial stirred-tank processes, and for packed-column processes, which have cycling times well over an hour [10]. Shorter cycling times result in more efficient utilization of the IEX groups on the membrane, which reduces the IEX capacity needed to achieve a fixed protein output compared to stirred-tank processes. Reduced capacity demands should translate into reduced capital costs for a new plant, especially if IEX membranes can be retrofitted into existing ultrafiltration membrane equipment. Based on these results, IEX membranes are a promising new method for whey protein isolation.

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AN EVALUATION OF THE DIFFERENTIAL PARTITIONING AND SEPARATION OF C_{60} AND C_{70} FULLERENES IN A BIPHASIC SYSTEM USING CENTRIFUGAL PARTITION CHROMATOGRAPHY (CPC)

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ABSTRACT

The partition coefficients of C_{60} and C_{70} fullerenes were measured in several different organic, 2-phase (liquid-liquid) systems using centrifugal partition chromatography (CPC). The partition coefficients of C_{60} and C_{70} were sufficiently different in some biphasic solvent systems to provide a CPC separation of these fullerenes. A phase diagram was made of the best 2-phase system for fractionating C_{60} and C_{70} fullerenes. This system contained the solvents 1,2-dichlorobenzene, isooctane, and dimethylformamide (DMF). The separation times, selectivity, and efficiency are affected by the ratios of the major solvent components, the addition of small amounts of a quaternary solvent, and the temperature (in addition to the usual instrumental parameters). Preparative separations of fullerenes were done and a maximum batch production was calculated for

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one solvent system. It appears that a significantly greater amount of fullerenes can be purified per run in this system than in corresponding HPLC systems. The rather unusual organic biphasic solvent systems developed for the fractionation of fullerenes may be useful for future separation and purification of more conventional organic compounds.

INTRODUCTION

Fullerene separations have been achieved in high performance liquid chromatography (HPLC) using several types of stationary phases. Over fifteen liquid chromatographic stationary phases have been evaluated including silica gel[1-3], alumina[4], graphite[5], monomeric and polymeric C_{18}[6-10], native and derivatized cyclodextrins[10-11], gel permeation[12-13], and aromatic charge transfer[14-21] stationary phases. To date C_{60}, C_{70}, higher fullerenes (up to C_{96}), fullerene isomers, fullerene derivatives, and most recently, metal complexed fullerenes have been successfully isolated chromatographically.[1-24] Most of the aforementioned stationary phases can baseline resolve C_{60} and C_{70} without much difficulty provided the optimum mobile phase composition for each stationary phase is used. The recent demand for larger quantities of fullerenes has led to the challenge of separating fullerenes on a preparative basis. One of the problems in the area of preparative purification of fullerenes is that they have limited solubilities in most HPLC eluents. Ruoff et. al. determined that only 0.043 mg/ml of C_{60} can be dissolved in n-hexane, 0.001 mg/ml in ethanol, and 0.00 mg/ml in acetonitrile.[25] Even toluene dissolves only 2.8 mg/ml of C_{60}.[25] Fullerenes are known to be most soluble in chlorinated benzenes, carbon disulfide, toluene, methylene chloride, and chloroform (in order of decreasing solubility).[25] The better fullerene solvents (CS_2, chlorinated benzenes, etc.) are not often used for HPLC because of their volatility, odor, viscosity, and toxicity. Although both polar and nonpolar stationary phases have been used for fullerene separations, the mobile phases are fairly limited to the less polar solvents. Consequently, even though a reversed phase stationary phase may be used, typical reversed phase mobile phases (hydro-organic solvents) cannot be used.

Many early attempts at chromatographically separating fullerenes on preparative basis involved overloading analytical (25 cm x 0.46 cm) and

semi-preparative (25 cm x 1.0 cm) length columns. In 1990, Hawkins and coworkers reported that their semi-preparative 3,5-dinitrobenzoylphenylglycine (DNBPG) column could only resolve 0.5 mg of fullerene material per unit injection.[14] Welch and Pirkle later reported that a 1.5 m x 5 cm preparative column containing the same DNBPG stationary phase would separate 100 mg of C_{60} and C_{70}.[19] In contrast, they indicated that this stationary phase is capable of normally separating 20 g of a soluble mixture of more "typical" organic compounds of the same selectivity. Herren et. al., in 1993, claimed to improve preparative separations for C_{60} and C_{70} fullerenes using a chemically bonded tetrachlorophthalimidopropyl-modified silica (TAPA).[17] Approximately 1 mg could be separated per injection. Most of the HPLC stationary phases evaluated for large scale fullerene purifications were found to be impractical because recovery yields were quite low. Other stationary phases were found to be very costly because of the large diameter and length columns needed in addition to the high cost of column hardware. Another problem with preparative chromatography is the irreversible adsorption and degradation of some fullerenes when they are associated with the stationary phase.

Recently, we did a comparison study of the selectivity, resolution, column deterioration, higher fullerene isolation, and preparative purification (loading ability) of C_{60} and C_{70} fullerenes using several commercial aliphatic and aromatic stationary phases.[10] The general trend found was that alkyl-chain bonded silica stationary phases seemed to be best for the analytical separation of fullerenes. Conversely, aromatic stationary phases were found to be better for purifying fullerenes on a preparative basis. Between 2.5 and 3 mg of C_{60} and C_{70} were resolved on the aromatic analytical columns (25 cm x 0.46 cm) before the solubility of the fullerenes in the sample solvent exceeded that of the mobile phase. Also irreversible adsorption of fullerenes or associated degradation products occurred on all of the columns tested. This results in significant decrease in column performance for both analytical and preparative applications.

Ideally, the goal of preparative chromatography is to produce the largest quantity product of the highest purity per unit time while keeping the production cost low. Column loadability (amount that can be injected per unit run), to a large extent, is dictated by the peak to peak separation or

α value.[26-27] The amount that can be injected affects the cost of purification and, therefore, must be maximized. Numerous factors affect band broadening in preparative HPLC from the physicochemical chromatographic parameters (i.e., the surface area of the stationary phase, the temperature, the number of theoretical plates, and the chemical nature of the mobile phase) to the type of distribution isotherm.[27] In general, if a compound cannot be separated in a single run under optimum conditions, the simplest and most routine method for improving the separation involves increasing the bed length of the column. Guiochon and co-workers have published a series of related studies on the theory and general optimization parameters for preparative liquid chromatography.[28-30]

Because of the solubility limitation of fullerenes in common HPLC eluents, it is possible that an optimum separation method for the purification of fullerenes would involve the principles of liquid-liquid extraction and incorporate the efficiency, resolving power, speed, and convenience of chromatography. There is a chromatographic technique that utilizes these principles. Centrifugal partition chromatography allows one to do a series of liquid-liquid extractions in the chromatographic mode. CPC is a variation of countercurrent chromatography. CPC has a liquid mobile phase and a liquid stationary phase. Consequently, problems with irreversible adsorption or degradation by a solid stationary phase are avoided. The liquid stationary phase is held in place by a centrifugal field while the liquid mobile phase is pumped through it. Detailed descriptions of the CPC apparatus and theory have been published.[31-34] Some of the more common two phase liquid systems used to separate organic compounds with the CPC apparatus are hydro-organic and water rich systems such as octanol/water and methanol/hexane/water.[35-37]

There are several advantages to using CPC over preparative liquid chromatography such as the increased sample capacity obtained due to the large stationary phase to mobile phase volume ratio and the elimination of irreversible retention by use of the dual-mode of elution.[31-38] Also, unlike other chromatographic methods, the efficiency of CPC increases at very high flow rates.[33] These characteristics makes CPC an ideal large-scale separation or purification method. The effect of analyte concentration and injection volume (as a means to increase mass load) in preparative CPC has been evaluated.[35] Unlike preparative or analytical HPLC, increasing the concentration of analyte injected (at constant volume) does not cause a

dramatic decrease in separation efficiency. Hence, in most cases, band broadening that occurs from column overloading in HPLC is not as significant in CPC. In order to purify large quantities of compounds by HPLC, immense preparative columns are required. The only limitation affecting mass load in CPC is the solubility of fullerenes in the mobile phase.

In this work, we examine several organic liquid-liquid, two phase systems and evaluate the partitioning behavior of fullerenes in them. Devising such two phase systems are not always straight forward since fullerenes are insoluble or very poorly soluble in many of the more popular chromatographic solvents (e.g., water, methanol, other alcohols, acetonitrile, etc.) that are used to form liquid biphasic systems. The fullerenes must be at least somewhat soluble in both liquid phases if the system is to be used for separations. In addition the partition coefficient (K) of various fullerenes between the liquid phases must be different. Also, for large scale separations, the fullerenes must be appreciably soluble in at least one of the phases. We demonstrate: (1) that fullerenes are amenable to such systems (2) that the selectivity of these systems can be altered or optimized, and (3) that both separations and partition data can be obtained in a liquid-liquid countercurrent chromatographic experiment.

EXPERIMENTAL

Materials. Isooctane, hexane, heptane, toluene, acetonitrile, and methyl-*tert*-butyl ether were of HPLC grade and obtained from Fisher (Pittsburgh, PA). Aldrich Chemical Company (Milwaukee, WI) supplied the dimethylformamide (DMF), dimethylsulfoxide (DMSO), N-methyl-2-pyrrolidinone (NMP), γ-butyrolactone, decalin, and 1,2-dichlorobenzene. Pure fullerene standards of C_{60} and C_{70} were purchased from either Polygon Enterprise (Waco, TX), Fluka (Ronkonkoma, NY), or provided by IBM at the Almaden Research Center (San Jose, CA).

Apparatus. All chromatographic experiments were performed on a Sanki Laboratories Inc. (Mount Laurel, NJ) Model CPC-NMF centrifugal partition chromatograph equipped with an adjustable temperature thermostat (from 15 to 35 °C). The following Shimadzu (Columbia, MD) equipment was also used: two LC-6A pumps, a SPD-6A UV/Vis variable wavelength spectrophotometric detector with preparative flow cell, and a

SCL-6B system controller. The system also included a Rheodyne injector and switching valve (models 7125 and 7010, respectively) equipped with a 1 ml injection loop. A Recorder Company 4500 series strip chart recorder (San Marcos, TX) was used to record the data. After elution, the C_{60} and C_{70} peaks were collected with an Isco Cygnet fraction collector (Lincoln, NE).

The rotor of the CPC apparatus holds up to 12 cartridges although either 3 or 6 cartridges were used in this study. A complete description of the cartridges and the CPC system was given previously.[32,35-36] Generally the cartridges are filled with the liquid stationary phase at high flow rates. The centrifugal spin rate is chosen which generates the centrifugal field and the mobile phase is slowly pumped through. When equilibrium is established, the ducts are filled with the mobile phase, the channels are filled with the stationary phase, and only the mobile phase exits the CPC apparatus.

The CPC apparatus can be used in the ascending or the descending elution mode. In the descending mode, the stationary phase is the upper or less dense liquid and the mobile phase is the more dense liquid phase. In this case, the more dense liquid (i.e., the mobile phase) flows through the stationary phase from the top of the apparatus to the bottom. When using the ascending mode the opposite occurs. The most dense liquid becomes the stationary phase and the less dense liquid is mobile phase which is pumped up through the instrument from the bottom to the top of the centrifuge. Both modes were used during the course of this study and are labeled accordingly in the appropriate tables and figures.

Procedure. Numerous organic liquid systems were evaluated with small 5 ml batch scale experiments to determine whether biphasic systems were formed and if they could be used as stationary and mobile phases in CPC. Approximately, 1 mg of fullerenes were dissolved in each liquid system and sonicated for 10 minutes. 10 µl of each layer was injected onto a liquid chromatograph equipped with a Astec C_{18} (25 cm x 0.46 cm) (Whippany, NJ) stationary phase. The peak areas of C_{60} and C_{70} in one phase were compared to the peak areas of C_{60} and C_{70} in the other phase. The biphasic system giving the largest fullerene concentration difference between the top and the bottom layers were further evaluated as stationary and mobile phases for CPC.

All biphasic ternary liquid systems used in CPC were mixed on a mechanical stirplate for 1 hour and then placed an additional 15 minutes in a ultrasound bath to ensure complete mutual saturation. The appropriate number of cartridges (i.e., the column in CPC) were filled at a high flowrate with the liquid stationary phase in the proper elution mode. With the centrifuge field spin rate between 700 and 1700 rpm in the opposite elution mode, the mobile phase was pumped in at a flowrate between 0.2-1.0 ml/min. The system was equilibrated for 1-2 hours or until no more stationary phase was displaced. The effluent is collected via a graduated cylinder so that the volume of stationary phase displaced can be measured. Each analysis required only 400 ml of solvent because the mobile phase was continuously recycled until the beginning of the first peak eluted. The most frequently used 2-phase organic system was the isooctane/dimethylformamide/1,2-dichlorobenzene. The more dense dimethylformamide/1,2-dichlorobenzene layer was used as the stationary phase while the less dense isooctane/1,2-dichlorobenzene mixture served as the mobile phase. While this unusual biphasic solvent system was determined to be an ideal system to differentially solubilize fullerenes, it is not the best system to use with this model CPC apparatus since it also tends to accentuate the erosion of the graphite disc located within the rotary seals. Optimum flowrates for this system were between 0.2 and 0.5 ml/min. Between 50 - 500 μl of a 5 or 15 mg/ml concentration of the C_{60} and C_{70} dissolved in 1,2-dichlorobenzene was injected. Detection was set at 384 nm because Guiochon and co-workers reported that C_{60} and C_{70} have the same extinction coefficients at that wavelength. [9]

The ternary phase diagram was determined by weighing specific amounts of each of the three liquids, vigorously shaking, and then allowing them to thermally equilibrate for 15 minutes. The solvent system was found to be biphasic by visual examination of the meniscus. The precise composition of each phase was ascertained by gas chromatography utilizing thermal conductivity detection.

RESULTS AND DISCUSSION

Table 1 lists several of the organic, biphasic solvent systems and experimental parameters that were varied to find an optimum liquid-liquid system to fractionate C_{60} and C_{70}. Many more solvent systems were prepared and evaluated than are shown in Table 1. However, little or no

Table 1. Partition Coefficient Determination of C_{60} and C_{70} in Various Solvent Systems

System number	Cartridge number	Elution mode	Fullerene Solvent Systems good % v-v	moderate % v-v	poor % v-v	poor % v-v	Temp (°C)	K1 C60	K2 C70	Vr1 C60 (ml)	Vr2 C70 (ml)	Vo (ml)	Vt (ml)	α
1	2	A	1 toluene	3 hept	2 DMF		22	0.96	0.96	46	46	24	47	1
2	3	D	2 toluene	1 hept	1 DMSO		20	3.13	3.13	178	178	15	67	1
3	3	D	1 deca		1 DMF		16	0.84	0.84	60	60	24	67	1
4	3	D	3 deca	2 toluene	2 DMF	2 DMSO	17	7.62	8.38	312	340	30	67	1.1
5	3	A	3 deca	3 toluene	2 DMF	2 DMSO	17	7.33	8.22	124	132	58	67	1.12
6	6	D	4 deca	1 toluene	4 DMF		18	7.53	7.93	695	730	40	127	1.05
7	3	D	11 deca	3 toluene	8 NMP	0.2%water	16	6	6	240	240	30	67	1
8	3	D	3 DCB	10 deca	8 DMF		17	0.41	0.46	120	110	30	67	1.12
9	3	D	2 DCB	8 deca	5 DMF		17	0.45	0.52	110	100	32	67	1.15
10	3	D	1 DCB	4 deca	4 DMF		18	0.16	0.18	255	230	29	67	1.15
11	6	D	1 DCB	6 isoo	4 DMF		19	2.08	2.27	77	73	30	127	1.09
12	3	D	1 DCB	4 isoo	2 DMF		15	2.33	2.7	43.5	41	26	67	1.16
13	3	A	1 DCB	4 isoo	2 DMF	0.1%water	17	2	2.43	116	137	18	67	1.22
14	3	A	1 DCB	4 isoo	2 DMF	0.5%water	17	2.17	2.67	123	147	17	67	1.23
15	3	D	1 DCB	4 isoo	2 DMF	0.8%water	17	2.13	2.33	76	72	30	127	1.09
16	3	D	1 DCB	4 isoo	2 DMF	1 MeCN	15	1.15	1.21	61	59	22	67	1.06
17	3	A	1 DCB	4 isoo	2 DMF	0.5%MTBE	15	2.23	2.75	114	132	22	67	1.2
18	3	A	1 DCB	4 isoo	2 DMF	1% MTBE	15	2.02	2.96	118	165	17	67	1.47
19	3	A	1 DCB	4 isoo	2 DMF	5% MTBE	15	2.29	2.82	103	118	39	67	1.23
20	3	A	1 DCB	4 isoo	2 DMF	1% MTBE	20	2.34	2.61	133	146	18	67	1.15

[a] A and D correspond to the ascending and descending mode of elution, respectively. In the ascending mode the stationary phase is the more dense liquid phase and the mobile phase is the less dense liquid layer. The opposite is true for the descending elution mode (See Experimental).

[b] The solvent that dissolves the greatest quantity of C_{60} and C_{70} in each system is refered to as a "good" fullerene solvent. "Poor" fullerene solvents do not solubilize C_{60} and C_{70}, whereas "moderate" solvents dissolve small amounts of C_{60} and C_{70}. The abbreviations are as follows: hept = heptane, DMF = dimethylformamide, DMSO = dimethylsulfoxide, deca = decalin, isoo = isooctane, MeCN = acetonitrile, MTBE = methyl-*tert*-butyl-ether, and DCB = 1,2-dichlorobenzene.

[c] The partition coefficients (K = [fullerenes]sp/[fullerenes]mp) were calculated with the liquid layer solubilizing the greatest quantity of fullerenes as the stationary phase.

[d] Vr_1 and Vr_2 are the retention volume in ml of C_{60} and C_{70}, respectively. V_o is the volume (in ml) of the mobile phase and Vt is the total volume in ml of the CPC apparatus (which is dependent upon the number of cartridges used).

[e] The selectivity factors were calculated by the following equation: $\alpha = K_2/K_1$.

fractionation of C_{60} and C_{70} was observed in these systems. A few examples of the "unsuccessful" systems are included in Table 1 for illustrative purposes (i.e., system numbers 1-3 and 7 in Table 1). In each biphasic, ternary liquid system, a well known "good" fullerene solvent is combined with a "moderate" and/or "poor" fullerene solvent. The liquid in each ternary solvent system that dissolves the greatest quantity of fullerenes is denoted as the "good" fullerene solvent. Examples of "good" fullerene solvents are 1,2-dichlorobenzene, decalin, and toluene. The "poor" fullerene solvents are polar aprotic solvents such as dimethylformamide and dimethylsulfoxide. "Poor" fullerene solvents are necessary in order to obtain a two phase system and to impart selectivity to the systems. It should be noted that other polar protic and aprotic solvents such as methanol and acetonitrile also can be used to form biphasic organic systems with "good" fullerene solvents. However, these systems are not included since there was little encouraging evidence of fullerene fractionation in the early studies mentioned previously. The liquid phase in which the fullerenes were most soluble (Table 1) was always used as the stationary phase.

We previously reported on the use of CPC to determine the partition coefficients of a variety organic compounds in many liquid-liquid two phase systems.[31] The basic CPC retention equation can be rearranged so that the partition coefficient is easily determined:

$$K = [(Vr - Vt)/ Vs] + 1 \qquad [1]$$

where Vr is the retention volume in ml of the compound of interest, Vt is the total internal volume of the CPC instrument in ml, Vs is the stationary phase volume in ml, and K is the partition coefficient of the compound between the stationary phase and the mobile phase. This equation applies to the ascending elution mode. Therefore, the reciprocal (1/K) is used to determine the partition coefficient when using the descending mode of elution.

The partition coefficients of C_{60} and C_{70} fullerenes in a large number of biphasic systems also are shown in Table 1. As can be seen from this data, the various solvent systems generated a wide variety of partition coefficients. Hence the partition coefficients of C_{60} and C_{70} can be varied over an order of magnitude if desired. The highest partition coefficient measured was 8.38 and the lowest was 0.16. There does not appear to be any

correlation between the size of the fullerene's partition coefficient (K) and their selectivity (i.e., the α-values in Table 1). For example, system 4 which yields larger partition coefficients does not necessarily provide any greater selectivity than system 12 which gives small partition coefficient values. Systems 1 through 12 are representative of the initial experiments in which we hoped to find a "highly" selective system for the fullerenes. The 1,2-dichlorobenzene/isooctane/DMF solvent combination in trial 10 afforded the largest selectivity value for any three component system tested. However, as can be seen from the data in Table 1, adding small amounts of additional components or modifiers can cause the α values to change. Hence attempts were made to improve the selectivity and/or efficiency by adding different minor components. For example, the 1,2-dichlorobenzene/isooctane/DMF biphasic system is sensitive to small amounts of water. In trials 14-16, the ratio of water was varied from 0.1 percent to just under 1 percent. No basic trends were observed when attempting to improve selectivity by adding either small or large increments of water. The optimum water amount was found to be 0.5 % and provided an α-value of 1.23. Small quantities of methyl-*tert*-butyl-ether was added to the 1,2-dichlorobenzene/isooctane/dimethylformamide system as well. A trend similar to that for water was observed. The optimum methyl-tert-butyl-ether content was found to be between 0.5 % and 5 %. 1 % of methyl-*tert*-butyl-ether added to the previously determined optimum biphasic system and gave the largest selectivity value in this study (system 18, Table 1). Methyl-*tert*-butyl ether was chosen because it greatly enhanced the separation efficiency of monoterpenes hydrocarbons in reverse phase liquid chromatography.[39] It was one of the few additives studied that also seemed to enhance the efficiency of the fullerene separation.

In some cases, changing the temperature appeared to alter the retention and selectivity of the fullerenes (Table 1). Unlike HPLC, the mobile and stationary phase in CPC can alter their composition with a change in temperature (see the ternary phase diagram in Figure 1). As discussed in the preceding paragraph, even a small change in solvent composition can significantly affect the elution of fullerenes. Consequently the influence of temperature on retention and selectivity (α) is not as straight-forward in CPC as it is in HPLC (where the stationary and mobile phase composition do not change with temperature). Since the solvent composition and temperature effects are coupled in CPC, there is no way to

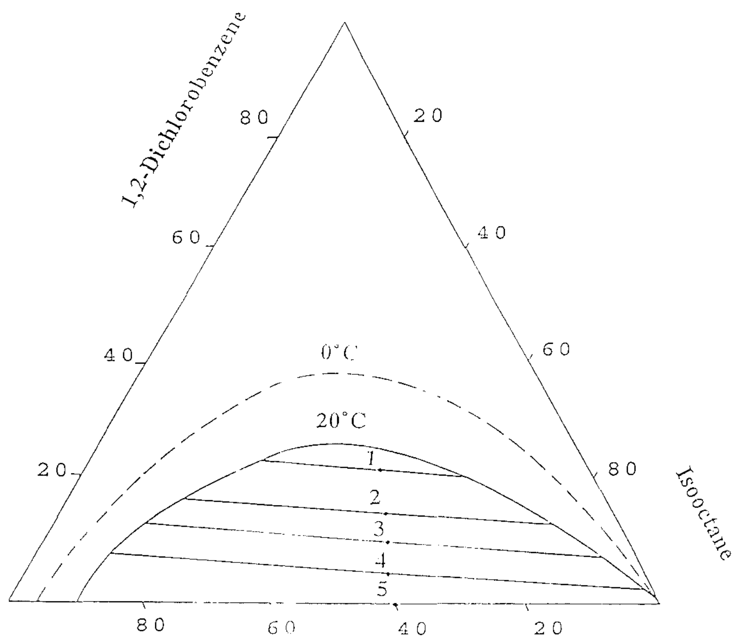

Figure 1. Ternary mass phase diagram for the isooctane/dimethylformamide/ 1,2-dichlorobenzene system at two temperatures, 0 °C the dotted curve and 20 °C the darkened curve. The region above the temperature curves indicates the miscible monophasic area and the region below designates the region where two layers are formed or the biphasic solution region. The tie lines were established for the 5 different chemical compositions shown in Table 2 at 20 °C.

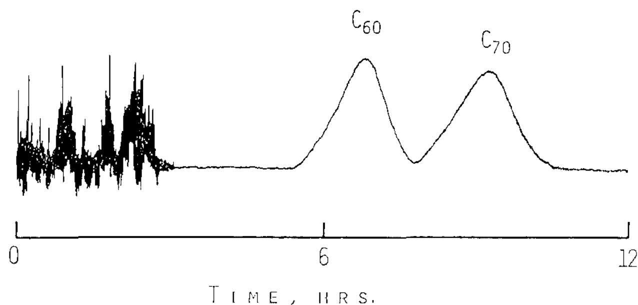

Figure 2. CPC ascending mode chromatogram showing the separation between C_{60} and C_{70}. The conditions were as follows: solvent system = 4:2:1 isooctane/DMF/DCB (v/v/v) + 1% MTBE; the isooctane-rich mobile phase ascended; flowrate 0.3 ml/min; rotation rate 1000 rpm; cartidge number 3; wavelength 384 nm; temperature 15 °C, injected volume 150 µl of a 15 mg/ml solution; and chartspeed 2 cm/hr.

predict *a priori* whether lowering the temperature will increase selectivity (α) and retention. However, the best separation in this work was obtained at 15 °C as opposed to higher temperatures (Experiment 18, Table 1, and Figure 2).

The ternary mass diagram for the isooctane/DMF/1,2-dichlorobenzene solvent system (the "optimum" system as determined from the data in Table 1) is found in Figure 1. The regions above the dashed and solid curved lines designates the monophasic area or homogeneous region (for the respective temperatures). Obviously these solvent compositions would be useless for any separation by countercurrent chromatography or liquid-liquid extraction. The region below the solid and dashed curved lines corresponds to the biphasic region which is suitable for the CPC apparatus. The dashed line designates the biphasic boundary at 0 °C and the solid line indicates the biphasic boundary at 20 °C. The tie lines are calculated according to the lever rule which is described in detail elsewhere.[37] These tie lines allow one to precisely quantitate the composition of the two phases obtained when ternary liquids become saturated with one another. As can be seen, the 1,2-dichlorobenzene partitions almost equally between the upper and lower phases. The exact compositions of the tie lines (in mass percentages or g/100g) of the two phase liquid-liquid mixtures are found in Table 2. The optimum solvent composition that was the focus of most of this study is indicated by point one. The composition is 46.4% isooctane, 31.7% DMF, and 21.9 % 1,2-dichlorobenzene (w/w).

The ternary phase diagram also shows that the waterless isooctane/DMF/1,2-dichlorobenzene system used is critically temperature dependent. It was observed that the two phases in this system become homogeneous at temperatures ≥ 30 °C. As noted in the experimental section, the lower (more dense) layer of this biphasic system consists mainly of DMF and 1,2-dichlorobenzene. The denser lower layer dissolves fullerenes better than the isooctane-rich upper layer. The fullerene partition coefficients (Ks) are higher than 2.0 (See Table 1). Therefore, when the two phases are equilibrated, the fullerene concentration is more than 2 times greater in the lower DMF-rich stationary phase than in the upper isooctane-1,2-dichlorobenzene mobile phase. When this system is warmed, the bottom layer increases in volume at the expense of the top layer. This decreases the difference in the C_{60} and C_{70} partition coefficients

Table 2. Chemical Composition of Ternary Phase Diagram Tie Lines

Point[a]	DCB % w/w	Isoctane % w/w	DMF % w/w	Solubilization Temperature °C[b]
1	21.9	46.4	31.7	31
2	15	51	34	56
3	10	53	37	60
4	5	56	39	67
5	0	60	40	75

[a] Numbers correspond to the those on Figure 1 (ternary phase diagram) Point 1 refers to the volume ratio 1:4:2 DCB/isooctane/DMF (v/v/v).

[b] The biphasic system becomes monophasic at the solublization temperature.

although both fullerenes are still more soluble in the lower DMF-1,2-dichlorobenzene phase. Temperature changes also are known to alter the physicochemical properties of liquids including their density, viscosity, vapor pressure, and the partition coefficients of dissolved solutes.[31,33,37-38] All of these factors previously have been shown to affect CPC separations.

A model has been developed by Cretier and Rocca which enables one to predict the maximum sample capacity on a given preparative silica based packed stationary phase.[40-41] We have used an analogous model for CPC.[35] It was extended so that one can estimate the maximum injection volume, V_{max}, in CPC, taking into account peak symmetry and the absence of mass overload by:

$$V_{max} = Vr_2 - Vr_1 - [(W_2 + W_1) / 2] \quad [2]$$

where Vr is the retention volume, W is the width of the peak at the base, and the subscripts 1 and 2 corresponds to C_{60} and C_{70}, respectively.[35] Using the retention volume data from Figure 2 and solving for V_{max}, one obtains:

$$V_{max} = 164.6 \text{ ml} - 117.6 \text{ ml} - [(37.8 \text{ ml} + 36.9 \text{ ml}) / 2] = 9.65 \text{ ml} \quad [3]$$

Therefore, the maximum amount that can be separated as indicated by equation 3 is 144.8 mg (9.65 ml x 15 mg/ml) of a fullerene solution with 100 % recovery and purity per run. The above calculation was done using data generated with 3 cartridges, however, up to 12 cartridges can be loaded

into the CPC apparatus. Therefore, theoretically the quantity of fullerenes can be further increased provided the pressure limits of the system are not exceeded and the allowed degree of peak overlap does not change.

CONCLUSIONS

It is possible to formulate a variety of different liquid, organic, biphasic solvent systems that can be used to fractionate fullerenes. When used in conjunction with centrifugal partition chromatography (a type of countercurrent chromatography) one can measure partition coefficients and carry out preparative-scale separations. The current CPC separation can purify approximately 50 times the amount of fullerenes (per batch) as compared to previously reported HPLC methods. The relative mildness of this technique could make it useful in isolating greater percentages of the more labile fullerenes such as those containing metals. These novel liquid-liquid systems may be useful for separating more conventional organic compounds as well.

ACKNOWLEDGEMENT

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(5), 1035-1036 (1995)

MEETING REPORT

SIXTEENTH INTERNATIONAL SYMPOSIUM ON CAPILLARY CHROMATOGRAPHY
September 27-30, 1994
Riva del Garda, Italy

The above symposium, which was held at the Palazzo dei Congressi in Riva, on the shores of Lake Garda, was a highly successful one. It was attended by about 700 conferees. The conference opened with a welcoming address by Professor Pat Sandra (Chairman), then the presentation of the Golay Award to Professor J.W. Jorgenson (well deserved), followed by an award address entitled, "Microcolumn Separations and Complex Mixture Analysis," which was clearly presented and well received.

The presentations were divided into plenary lectures, oral presentations, workshops, seminars and poster presentations. There were a total of 37 lectures, dealing with different microcolumn separations (GC, LC, CE and SFC). Over 370 posters were divided into a) General Theory; b) Column Technology - Stationary Phases; c) Environmental Applications; d) Natural Products and Food Research; e) Biomedical and Pharmaceutical Applications; f) Industrial Applications; g) Sampling and Trace Analysis; h) Multidimensional and Hyphenated Techniques; i) Micro LC and Applications; j) SFC and Applications, k) SFE and Applications; l) Electromigration Methods and Applications; and m) Last Minute Contributions.

The combination of a few lectures in a session allowed ample time for poster viewing and discussion among the conferees. Overall, the meeting was very successful and Professor Sandra and the Scientific Committee and organizers should be commended on a job well done.

Next year's conference will be held in the United States on May 7-11, 1995 at Wintergreen in a most beautiful setting in Virginia. For information, contact the Symposium Coordinator, Joy Wise, PO Box 4153, Frederick, Maryland 21705-4153 or Dr. Milton L. Lee (Chairman), Brigham Young University, Provo, Utah 84602-4672. All those who are

interested in microseparations, be it GC, HPLC, SFC, SFE or CE should attend this important scientific and truly international conference.

The 18th Conference will be held again in Riva on May 20-24, 1996.

Reviewed by
Haleem J. Issaq, Ph.D
Associate Editor

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(5), 1037-1038 (1995)

THE BOOK CORNER

ADVANCES IN CHROMATOGRAPHY, Volume 34, Edited by P.R. Brown and E. Grushka, Marcel Dekker, Inc., New York, NY, 456 pages, 1994. Price: $165.00.

The present volume of this excellent and prestigious series is made up of seven chapters, written by experts in their respective areas. Although the series is entitled, "Advances in Chromatography," Volume 34 contains two chapters dealing with capillary electrophoresis (CE), which is an electromigration separation technique. In the future, we will probably see more and more reviews dealing with this new and powerful microseparation technique with an efficiency exceeding one million theoretical plates. The two CE chapters selected deal with very important topics: serum and plasma proteins, and carbohydrates. These two reviews give a wealth of information and are well written and illustrated.

The other 5 chapters are well balanced: Chapters 3 and 7 are theoretical representations of peak overlap and optimization of quantitative analysis in separation science. Chapter 2 deals with analysis of natural products, which is a very interesting and important topic, considering the fact that many modern drugs and pharmaceuticals are the result of natural products research. Another interesting topic is discussed in Chapter 5, which deals with environmental applications of supercritical fluid chromatography, a technique which did not perform commercially and is not as well accepted as it should have been. However, its use in the analysis of environmental samples is useful and should be encouraged. Chapter 6 deals with the HPLC separation of organic ions. A discussion of reversed phase, ion-exchange, and ion-exclusion is given, with excellent illustrations including figures, tables and up-to-date references.

Volume 34 is a welcome addition to the separation science library. In light of the success of CE, may we recommend to the editors to change the title of the series to "Advances in Chromatography and Electrophoresis."

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1. **High-Performance Capillary Electrophoresis of Human Serum and Plasma Proteins**, O. W. Reif, R. Lausch and R. Freitag, (1)

2. **Analysis of Natural Products by Gas Chromatography/Matrix Isolation/Infrared Spectrometry**, W. M. Coleman III and B. M. Gordon, (57).

3. **Statistical Theories of Peak Overlap in Chromatography**, J. M. Davis, (109).

4. **Capillary Electrophoresis of Carbohydrates**, Z. El Rassi, (177).

5. **Environmental Applications of Supercritical Fluid Chromatography**, L. J. Mulcahey, C. L. Rankin and M. E. P. McNally, (251).

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JOURNAL OF LIQUID CHROMATOGRAPHY, 18(5), 1039 (1995)

ANNOUNCEMENT

BASIC PRINCIPLES OF HPLC and HPLC SYSTEM TROUBLESHOOTING

One-Day & Two-Day In-House Training Courses

The courses, which are offered for presentation at corporate laboratories, are aimed at chemists and technicians who work with HPLC. They cover HPLC fundamentals and method development, as well as systematic diagnosis and solution of HPLC module and system problems.

The following topics are covered in depth:

- Introduction to HPLC Theory
- Modes of HPLC Separation
- Developing and Controlling Resolution
- Mobile Phase Selection & Optimization
- Ion-Pairing Principles
- Gradient Elution Techniques
- Calibration & Quantitation
- Logical HPLC Troubleshooting

The instructor for the courses, Dr. Jack Cazes, is Editor-in-Chief of the Journal of Liquid Chromatography, of Instrumentation Science & Technology journal, and of the Chromatographic Science Book Series. He has been intimately involved with liquid chromatography for more than 30 years; he pioneered the development of modern HPLC technology. Dr. Cazes was also Professor-in-Charge of the ACS short course and the ACS audio course on Gel Permeation Chromatography for many years.

Details of these in-house courses may be obtained from Dr. Jack Cazes, Post Office Box 2180, Cherry Hill, NJ 08034-0162, USA; Tel: (609) 424-3505; FAX: (609) 751-8724.

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(5), 1041-1046 (1995)

LIQUID CHROMATOGRAPHY CALENDAR

1995

FEBRUARY 13 - 15: PrepTech '95, Sheraton Meadowlands Hotel, East Rutherford, New Jersey. Contact: Dr. Brian Howard, ISC Technical Conferences, Inc., 30 Controls Drive, Shelton, CT 06484-0559, USA.

MARCH 6 - 10: PittCon'95: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, Louisiana. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

APRIL 2 - 7: 209th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

MAY 21: Techniques for Polymer Analysis and Characterization, a short course, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

MAY 22 - 24: 8th International Symposium on Polymer Analysis and Characterization, Sanibel Island, Florida. Contact: ISPAC Registration, 815 Don Gaspar, Santa Fe, NM 87501, USA.

APRIL 25 - 28: Biochemische Analytik '95, Leipzig. Contact: Prof. Dr. H. feldmann, Inst. fur Physiologische Chemie der Universitat, Goethestrasse 33, D-80336 Munchen, Germany.

MAY 23: Miniaturization in Liquid Chromatography versus Capillary Electrophoresis, Pharmaceutical Institute, University of Ghent, Ghent, Belgium. Contact: Dr. W. R. G. Baeyens, Univ of Ghent, Pharmaceutical Inst, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 28 - JUNE 2: HPLC'95, 19th International Symposium on Column Liquid Chromatography, Convention Center, Innsbruck, Austria. Contact: HPLC'95 Secretariat, Tyrol Congress, Marktgraben 2, A-6020 Innsbruck, Austria.

MAY 31 - JUNE 2: 27th Central Regional Meeting, ACS, Akron Section. Contact: J. Visintainer, Goodyear Research, D415A, 142 Goodyear Blvd, Akron, OH 44236, USA.

JUNE 6 - 8: 28th Great Lakes Regional ACS Meeting, LaCrosse-Winona Section. Contact: M. Collins, Chem. Dept., Viterbo College, La Crosse, WI 54601, USA.

JUNE 11 - 14: 1995 International Symposium and Exhibit on Preparative Chromatography, Washington, DC. Contact: Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

JUNE 13 - 16: Capillary Electrophoresis, Routine Method for the Quality Control of Drugs: Practical Approach (in English); L'Electrophorese Capillaire, Methode de Routine pour le Controle de Qualite des Medicaments: Approche Pratique (in French), Monpellier, France. Contact: Prof. H. Fabre, Lab. de Chimie Analytique, Inst. Europeen des Sciences Pharmaceutiques Industrielles de Montpellier, Ave. Charles Flahault, 34060 Montpellier Cedex 1, France.

JUNE 14 - 16: 50th Northwest/12th Rocky Mountain Regional Meeting, ACS, Park City, Utah. Contact: J. Boerio-Goates, Chem Dept, 139C-ESC, Brigham Young Univ, Provo, UT 84602, USA.

JULY 9 - 15: SAC'95, The University of Hull, UK, sponsored by the Analytical Division, The Royal Society of Chemistry. Contact: The Royal Society of Chemistry, Burlington House, Picadilly, London W1V 0BN, UK.

JULY 7 - 8: FFF Workshop, University of Utah, Salt Lake City, UT. Contact: Ms. Julie Westwood, FFF Research Center, Dept. of Chem., University of Utah, Salt Lake City, UT 84112, USA.

JULY 10 - 12: FFF'95, Fifth International Symposium on Field-Flow Fractionation, Park City, Utah. Contact: Ms. Julie Westwood, FFF Researcvh Center, Dept. of Chem. Univ. of Utah, Salt Lake City, UT 84112, USA.

AUGUST 20 - 25: 210th ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 4 - 7: 13th International Symposium on Biomedical Applications of Chromatography and Electrophoresis and International Symposium on the Applications of HPLC in Enzyme Chemistry, Prague, Czech Republic. Contact: Prof. Z. Deyl, Institute of Physiology, Videnska 1083, CZ-14220 Prague 4, Czech Republic.

SEPTEMBER 12 - 15: 5th International Symposium on Drug Analysis, Leuven, Belgium. Contact: Prof. J. Hoogmartens, Inst. of Pharmaceutical Sciences, Van Evenstraat 4, B-3000 Leuven, Belgium.

OCTOBER 18 - 21: 31st Western Regional Meeting, ACS, San Diego, Calif. Contact: S Blackburn, General Dynamics, P. O. Box 179094, San Diego, CA 92177-2094, USA.

OCTOBER 22 - 25: 25th Northeastern Regional Meeting, ACS, Rochester, New York. Contact: T. Smith, Xerox Corp, Webster Res Center, M/S 0128-28E, 800 Phillips Rd, Webster, NY 14580, USA.

NOVEMBER 1 - 3: 30th Midwestern Regional ACS Meeting, Joplin, Missouri. Contact: J. H. Adams, 1519 Washington Dr., Miami, OK 74354-3854, USA.

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NOVEMBER 29 - DECEMBER 1: Joint 51st Southwestern/47th Southeastern Regional Meeting, ACS, Peabody Hotel, Memphis, Tenn. Contact: P.K. Bridson, Chem Dept, Memphis State Univ, Memphis, TN 38152, USA.

DECEMBER 17 - 22: 1995 International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

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FEBRUARY 26 - MARCH 1: PittCon'96: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, Chicago, Illinois. Contact: Pittsburgh Conference, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-9962, USA.

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

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

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

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

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OCTOBER 16 - 19: 52nd Southwest Regional ACS Meeting, Houston, Texas. Contact: J. W. Hightower, Dept. Chem. Eng., Rice University, Houston, TX 77251, USA.

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NOVEMBER 6 - 8: 31st Midwestern Regional Meeting, ACS, Sioux Falls, South Dakota. Contact: J. Rice, Chem Dept, S. Dakota State Univ, Shepard Hall Box 2202, Brookings, SD 57007-2202, USA.

NOVEMBER 9 - 12: 48th Southeast Regional ACS Meeting, Greenville, South Carolina. Contact: H. C. Ramsey, BASF Corp., P. O. Drawer 3025, Anderson, SC 29624-3025, USA.

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